Hepatitis virus infection in Libya.

Saleh, Mohamed

HEPATITIS VIRUS INFECTION IN LIBYA

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MOHAMED GIBRIAL SALEH

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ABSTRACT

The nature and prevalence of hepatitis B, C, D and GB virus infection was investigated in 301 "healthy" (control) subjects (including 76 blood donors), 16 patients with chronic liver disease (CLD) and 36 patients undergoing haemodialysis (HD) for chronic renal failure, from three areas in Libya. Overall, 112 (37.2%) control subjects were found to have serological markers of hepatitis B (HBV) or C (HCV) virus exposure, with 35 seropositive for HBsAg and 15 for HCV-RNA by polymerase chain reaction (PCR). Four HBsAg-positive subjects (including three blood donors) had active viral replication (HBV-DNA positive). 32 (88.9%) HD patients had evidence of HBV and/or HCV exposure, with 18 having current infections. Ten CLD patients had current infection with HCV and four with HBV. Results were correlated with demographic, clinical, and serum biochemical parameters and with histologically assessed severity of liver disease.

Genotyping of HCV in Libyan subjects, by restriction fragment length polymorphism, type-specific priming, nucleotide sequencing (of the 5'-untranslated, core and NS5 regions of the HCV genome) and phylogenetic analysis, was compared with genotypes in 106 patients with chronic hepatitis C from other parts of the world typed concurrently. Genotype 4 (with up to six distinct subtypes: a-f) was identified in 84% of the HCV-infected Libyans. GB virus infection was detected (by PCR using primers derived from the GBV-C genome) only in four (all HD patients), compared with 36 (33.9%) of the non-Libyans (in whom GBV infection correlated strongly with intravenous drug use). Overall, the findings indicate: (1) a high background prevalence of HBV and HCV infection in Libya (due at least partly to inadequate screening of blood donors) but very low prevalence of hepatitis D and GB viruses; (2) HCV genotype 4 predominates in Libya. Additionally, it is believed for the first time, HCV genotype 5 was identified in West Africa and the Caribbean.

DEDICATION

This Thesis is dedicated

to

my wife Suaad

my lovely daughters Norris, Ainour, Norhan

my son Ahmed

and

to my parents

for the support and patience they showed all through the duration of the study.

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CHAPTER 1

INTRODUCTION

1.1 CHRONIC LIVER DISEASE IN LIBYA

Until records of hospital admissions began to become available in 1959, very little was known about the incidence or causes of liver disease in Libya. Malaria, *Entamoeba histolytica*, and roundworm infections were known to be prevalent and echinococcosis (hydatid disease) was recognized to be endemic and enzootic. Indeed, Casoni [1911] conducted the work leading to development of his well known skin test for *Echinococcus* in Tripoli, while Cicogna [1961] recorded that animals in Libya are much infected with Echinococcus, with up to 60% of dogs, 40% of sheep, 70% of cattle and 20% of pigs examined being found to be infected. During 1951-1961, 57 patients were documented as having been treated for hepatic hydatid disease in the government hospital in Tripoli (Elhamel and Murthy, 1986; Aboundaya, 1986; Kalani et al. 1984), while Dar and Taguri, (1978) reported 180 surgical cases seen in Benghazi between 1971-1976. Nonetheless, there is still a paucity of reliable epidemiological data.

Schistosomiasis is also endemic, mainly in southern Libya (Sebha-Fezzan) where, for example, a prevalence of 86% has been recorded in the Wadi el Shati (Gebreel et al. 1985). Foci of infection also exist in the northern coastal region (Tripolitanio), principally in Tauroga (50 km south of Misurata), and in the eastern coastal region (Cyrenaica) mainly in the environs of Derna. The schistosome species involved are *S. haematobium*, which is predominant, and *S. mansoni*.

Although the majority of patients chronically infected with *S. mansoni* or *S. haematobium* do not have clinically significant liver disease, about 10% develop hepatosplenic schistosomiasis and a proportion of these progress to severe, decompensated chronic liver disease (Gilles, 1982; Prata, 1982). The resulting morbidity and mortality has major social and economic implications for a country such as Libya where such a sizeable population are infected with this parasite (Baccush et al. 1993). The variability in outcome of the infection has never been satisfactorily explained, but it is not unreasonable to presume that factors other than the parasite itself may be involved and that the simultaneous presence of two or more agents capable of damaging the liver are likely to lead to greater severity of tissue injury. Excessive alcohol consumption is the most obvious candidate but there has been no evidence that this accounts for the majority of cases of severe hepatosplenic schistosomiasis

and is highly unlikely in a strict Islamic country such as Libya. Given that viral hepatitis is endemic in North Africa, superimposed hepatitis viral infections are more likely to be involved. Indeed, studies in Egypt had suggested a correlation between infection with the hepatitis B virus and the development of severe hepatic schistosomiasis (Bassily et al. 1983), and recently completed studies in the Institute of Liver Studies at King's College Hospital, London, have since shown that almost all such patients in Brazil have chronic hepatitis B or C virus infections (Pereira et al. 1994; Pereira et al. 1995)

In Libya, there has been a steady increase in reported cases of presumed viral hepatitis since records began in 1959. However, since 1989, following strengthening of the regulations relating to notification of viral hepatitis, there has been a dramatic increase in the number of cases reported (Fig. 1.1), to the point where a total of 8562 were notified in 1993, i.e. an incidence of about 1:1000 of the total population and about 1:500 of urban dwellers (Secretary of Health in Libya, 1993). Details of the ages and sexes of infected individuals are not available.

In 1976, when Christie et al (1976) reported on a severe outbreak of viral hepatitis among pregnant women , some information on the nature of the infecting agent(s) began to become available. In that study, it had been possible to test only for hepatitis B virus (HBV) and, although some cases of HBV infection were identified, this virus could not be implicated in the high mortality rate (13%) associated with the epidemic. A similar outbreak in 1970, also in Tripoli (reported retrospectively by Wyatt (1977), documented a mortality rate of 26% but failed to identify the causative agent. In 1983, Gebreel and Christie (1983) reported that only 26.2% of Libyan patients with clinically diagnosed chronic viral hepatitis had HBV, and presumed that the remaining 73.8% had non-A, non-B viral infections. This impression was confirmed by Toweir et al. (1989) in a clinico-epidemiological study in 164 patients.

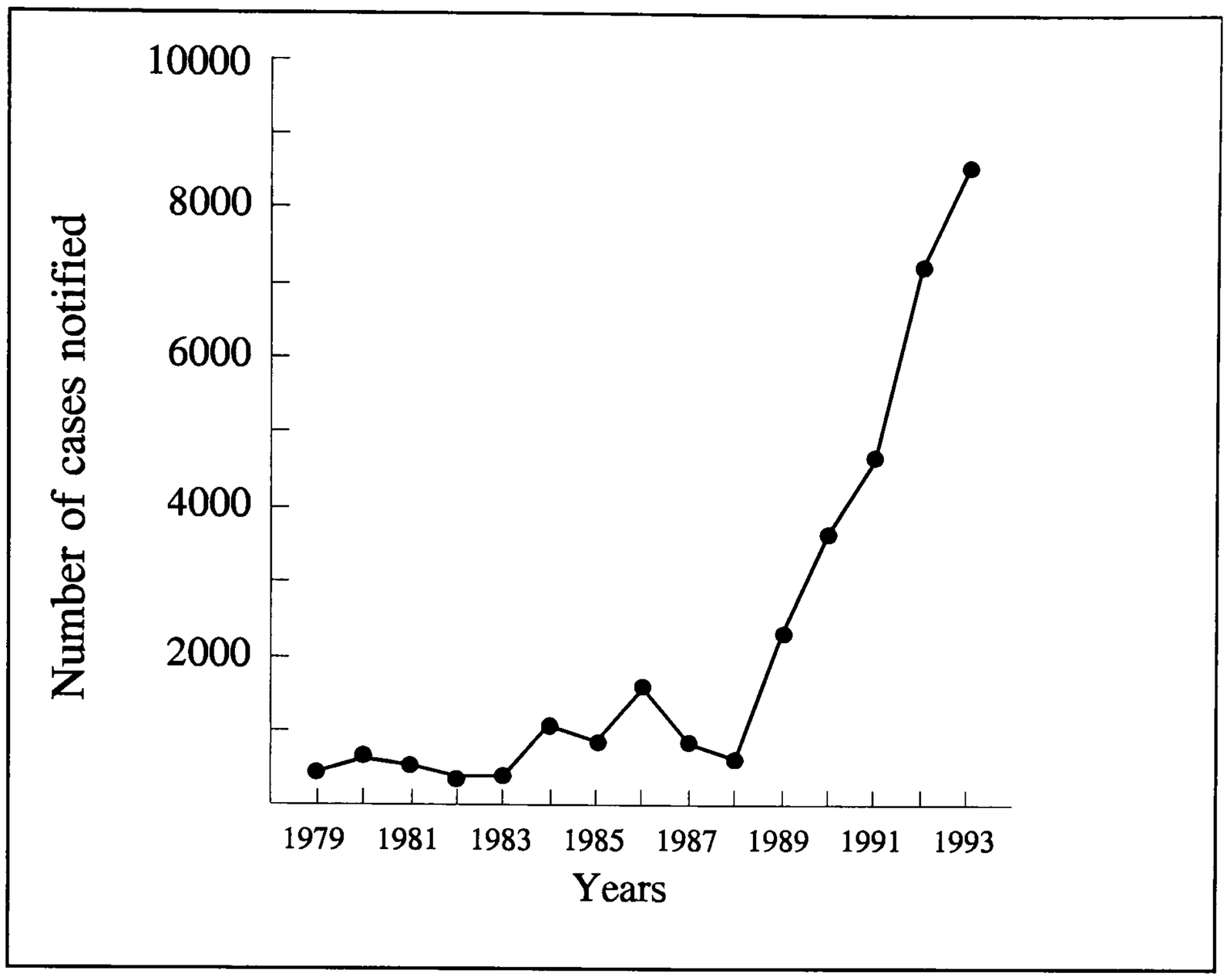


Figure 1.1 **Incidence of notified cases of viral hepatitis in Libya.**
Adapted from Libyan Secretary of Health Report [1959-1993].

Against this background, the present study was originally designed to investigate whether concomitant chronic infections with the hepatitis B or C viruses might be related to the development of severe hepatosplenic schistosomiasis in Libya. As controls for the study, sera were collected from a large number of apparently healthy individuals and other subjects without evidence of schistosomiasis. This had only just been completed when, unfortunately, the United Nations imposed sanctions against Libya. Direct travel between Libya and the United Kingdom was prohibited and the collection and transportation of further frozen samples to the U.K. became impracticable. However, as there were no definitive data on the prevalence of hepatitis viruses in Libya, it was decided to use the material collected to study hepatitis B, D and, particularly, hepatitis C virus infections in this group of subjects representing a cross-section of the Libyan population at large.

1.2 VIRAL HEPATITIS

That hepatitis can be caused by an infectious agent has been recognized since antiquity and, in early times, was known as "epidemic jaundice". The oldest written record of epidemic jaundice is believed to have been made by Hippocrates in the fifth century BC. A description of jaundice has also been found in the ancient Chinese literature around the year 200 AD, and what are thought to be references to jaundice have been noted in Egyptian and Indian Ayurvedic texts and in the Babylonian Talmud. In the eighteenth century attempts were made to explain jaundice on the basis of common bile duct obstruction and this became regarded as the principal cause. This concept proved to be an impediment that hampered understanding of the basic pathology underlying the development of jaundice. It was not until the late nineteenth century that the concept of jaundice resulting from a generalized infection of the liver parenchyma via the blood stream was suggested. It was also recognized at this time that jaundice (hepatitis) could occur in both sporadic and epidemic form and that it could be closely associated with acute and subacute necrosis of the liver (reviewed by Zuckerman, 1983b).

Understanding of viral hepatitis received considerable impetus from observations made during and immediately after World War II in military personnel contracting hepatitic illnesses. Clinical observations established that there were probably at least two agents capable of causing hepatitis: (1) a short incubation form, hepatitis A, which was variously described as infectious hepatitis, campaign jaundice, jaunisse de champs, or soldatengelbsucht, which appeared to be transmitted by the faecal-oral route and which was responsible for epidemics of hepatitis, and (2) a longer incubation form, hepatitis B (also termed serum hepatitis or homologous serum jaundice) that appeared to be blood-borne [for review see Krugman et al. (1979)]. The identification of filtrable agents capable of transmitting hepatitis to human volunteers established that these forms of hepatitis indeed had a viral aetiology [reviewed by Zuckerman, 1983a)

The first clues as to the nature of the hepatitis B virus came serendipitously from studies by Blumberg and colleagues in the U.S.A. who were investigating genetic differences in circulating low density serum lipoproteins using, as reagents, sera from multiply transfused haemophiliacs who had developed antibodies to lipoproteins (Blumberg et al. 1962). They

noted that a serum sample from an Australian aborigine gave a precipitin reaction by Ouchterlony immunodiffusion with sera from two haemophiliacs. This did not appear to be due to reaction of a lipoprotein with its corresponding antibody because the precipitin line stained strongly for protein but only weakly for lipid (Blumberg et al. 1965). The antigen in this precipitin line was designated "Australia antigen" and was later shown to be a protein associated with the hepatitis B virus (Blumberg et al. 1970)

Following the identification of the hepatitis A virus (HAV) a few years later (Feinstone et al. 1973), and with the availability of serological tests for infections with HAV and the hepatitis B virus (HBV), it became clear that there was at least one other major virus capable of causing hepatitis. This gave rise to the concept of "non-A, non-B" (NANB) viral hepatitis, although the term "NANB hepatitis" was sometimes used during the late 1970s and early 1980s as a catch-all category for what was also described as "idiopathic" or "cryptogenic" liver disease, often including patients with autoimmune ("lupoid") hepatitis (McFarlane and Williams, 1996).

In 1977 a new viral antigen was identified in the nuclei of hepatocytes of patients infected with HBV (Rizzetto et al. 1977). Subsequent studies revealed that this antigen was related to a new virus, later termed hepatitis delta virus (HDV), but it was soon apparent that this was not the cause of NANB hepatitis (NANBH) because HDV was always associated with HBV infection (Rizzetto, 1983). It was not until 1989 that Choo and colleagues at the Chiron Corporation in California, in collaboration with Bradley and colleagues at the Center for Disease Control in Atlanta, Georgia, identified the hepatitis C virus (HCV) as the major cause of blood-borne NANB viral hepatitis (see below). Since then, a number of other hepatitis viruses have been identified. One of these, the water-borne hepatitis E virus (HEV), has been responsible for large epidemics of hepatitis in India and the Kirghiz republic and for outbreaks in Nepal and South East Asia, including Burma, affecting mainly young and middle age adults and with a very high mortality (20-39%) in infected pregnant women(Kane et al. 1984). Outbreaks of HEV and sporadic infections have also been reported in North Africa, eastern Sudan, Somalia, the Ivory Coast, and Mexico, as well as in travellers returning from these areas (Centres for Disease Control, 1987a; Centres for Disease Control, 1987b). It has also been suggested that there may be a hepatitis F virus, causing sporadic fulminant hepatic failure(Fagan and Williams, 1990), and evidence is accumulating about a hepatitis G virus

(designated GB) which seems to be a major cause of "non-A-E" hepatitis (Zuckerman, 1995).

Nonetheless, epidemiologically HAV, HBV and HCV remain the three most important hepatitis viruses. From the clinical and socio-economic standpoint, the major difference between these three viruses is that chronic infections with HAV are rarely (if ever) seen whereas both HBV and HCV lead to chronic infection in a variable proportion of cases (see below). It is estimated that these two viruses each account for some 300 million individuals with chronic hepatitis viral infections worldwide (WHO, 1992). There is also increasing evidence that HBV and HCV may have oncogenic potential and may be strongly associated with the development of hepatocellular carcinoma, the highest incidence of which is in the developing countries where these viruses are endemic (Maupas et al. 1977; Coursaget et al. 1992; Coursaget et al. 1990b; Goudeau et al. 1979; Kew, 1994; Kew et al. 1990; Bukh et al. 1993a)

1.2.1 Clinical features of viral hepatitis

The clinical features of acute hepatitis due to infection with the various hepatitis viruses are similar in many respects, but are variable and cannot be used to distinguish between the different types of viral hepatitis. Following an incubation period of anything from a few days to several months, there is a pre-icteric phase lasting for 2-14 days. The initial symptoms are usually malaise (variously reported as lethargy or easy fatigueability) and anorexia, followed shortly by nausea, vomiting, and abdominal discomfort (often with right upper quadrant pain). These symptoms are relatively non-specific and a definitive diagnosis is seldom made during this period. Documenting the incubation period is rarely helpful because, although on average this is 25 days for HAV and 75 days for HBV and is intermediate (50 days) for NANBH (Krugman and Gocke, 1978; Zuckerman, 1986), much shorter (4 days to 2 weeks) (Craske et al. 1975) or longer (up to 33 weeks) (Zuckerman, 1988) incubations have been reported for NANBH.

About 10-15% of persons with acute viral hepatitis develop a serum sickness-like syndrome during the late incubation period or pre-icteric phase. This is characterized by one,

two or all three symptoms of a triad comprising a rash (usually urticarial), low grade fever, and polyarthralgia or arthritis. These symptoms are usually short lived and disappear with the onset of the icteric phase. The latter begins with the appearance of dark urine (and often pale stools) and deepening jaundice. These classical symptoms of acute viral hepatitis occur with similar frequency in all types of hepatitis (Dienstag and Purcell, 1977; Zuckerman, 1988).

The convalescent period following acute viral hepatitis starts with clearance of jaundice and cessation of the major symptoms, but weakness and easy fatigueability can last for some time after apparent recovery. The speed of recovery from an acute illness does not differ between the various forms of viral hepatitis (Norkrans, 1978), but can depend on whether the patient develops fulminant hepatitis or chronic infection ensues. About 1% of patients with acute HAV or HBV infections develop fulminant hepatic failure, which is associated with a high mortality. Fulminant hepatitis due to HCV has been reported in Japan(Muto et al. 1990) and Taiwan (Chu et al. 1994), but in other countries it seems to be very rare (Wright et al. 1991; Sallie et al. 1994). Chronic sequelae to acute HBV and HCV hepatitis occur with variable frequency (see below) but chronic HAV infection seems not to occur, although protracted or recurrent illnesses have been reported (Dusheiko and Zuckerman, 1994).

However, it is clear from epidemiological studies based on serological markers of these infections that very many patients have an anicteric illness which often goes unnoticed. This is particularly true of hepatitis C (see below) - the large majority of HCV-infected individuals being unable to recall having had a hepatitic illness (Cordoba et al. 1994). This may be because, for reasons as yet unknown, HCV infections tend to have a milder and generally more fluctuating course than acute or chronic infections with the other hepatitis viruses (Cordoba et al. 1994). Furthermore, the evolution of chronic HCV infection seems to be very slow and may often become apparent only incidentally through the finding of abnormal biochemical liver tests during routine health screening (Alter et al. 1989).

1.3 THE HEPATITIS B AND D VIRUSES

1.3.1 Biology of the hepatitis B virus

The hepatitis B virus (HBV) is a double-shelled DNA virus. The complete infectious virion is a 42 nm particle that was first identified by Dane and co-workers (Dane et al. 1970) and became known as the Dane particle. In 1980, Marion and colleagues described an HBV-like virus in Beechy ground squirrels (*Spermophilus beecheyi*) and proposed the type designation Hepadnaviridae (Marion et al. 1980). HBV is the prototype member of this new family which now includes several other, non-human, DNA viruses with similar features but distinct host specificities (Siddiqui et al. 1981). These include the Woodchuck hepatitis virus (WHV) (Summers et al. 1978) isolated from the eastern Woodchuck (*Marmota monax*), the ground squirrel hepatitis virus (GSHV) (Marion et al. 1980) that infects Beechy ground squirrels, duck hepatitis virus (DHV) (Mason et al. 1980) isolated from Pekin ducks (*Anas domesticus*), tree squirrel hepatitis virus (TSHV) (Feitelson et al. 1986) and a heron hepatitis virus (HHV) that has been isolated from grey herons (*Ardea cinerea*) (Sprengel et al. 1988). Other candidate members of the group include kangaroo hepatitis virus (KHV) and stink snake hepatitis virus (SSHV) (Bukhari and Tsiquaye, 1990).

Man is the principal host for HBV but it seems that the virus can infect some other higher primates such as chimpanzees, gibbons, orangutans

(Vaudin et al. 1988). The various other mammalian and avian hepatitis viruses each have similarly narrow host ranges, but are useful models for the study of HBV. Although marked liver tropism is a feature of all hepadnaviruses, HBV is harboured (and replicates) at numerous extrahepatic sites, including bone marrow, peripheral blood mononuclear cells, lymph nodes, spleen, kidney, colon, and even the skin and testes (Korba et al. 1986; Lieberman et al. 1987; Mason et al. 1993). But a very recent report suggests that the finding of HBV-DNA in peripheral blood mononuclear cells may be explained by adsorption of the virus on the surfaces of the cells (Kock et al. 1996).

During the early studies of Australia antigen a second antigenic specificity, termed Au2, was identified with an antiserum produced in rabbits immunized with serum from a patient with leukaemic reticulo-endotheliosis (Le Bouvier, 1971). Shortly afterwards, Le Bouvier and

colleagues described a group-specific antigenic specificity which was designated "*d*", and two mutually exclusive determinants, "*d*" and "*y*" - all isolates of the virus being either "*ad*" or "*ay*" (Le Bouvier et al. 1972). This was followed by identification of two additional specificities, "*w*" and "*r*" by Bancroft et al [1972]. These antigenic subtypes did not appear to have relevance of the virulence of the virus but were found to be useful epidemiological markers that seemed to correlate with severity of liver disease in different geographical areas (Tiollais et al. 1981).

Extensive studies over the past 25 years have since established the structure and genomic organization of HBV. The 42 nm particle is composed of an outer protein shell (the envelope) surrounding an inner protein shell (the core or capsid) which comprises a core protein and an associated HBV-specific DNA polymerase and encloses the viral DNA. The latter is a small, partially double-stranded circular DNA of between 3.2 and 3.3 kilobases (kb) and an estimated molecular weight of 1.6×10^6 to 2.0×10^6 (Howard, 1986; Ganem and Varmus, 1987). The genome consists of a long (L) strand of negative polarity and a short (S) strand of positive polarity. Most authorities describe the genomic organization by reference to a cleavage site for the restriction enzyme *Eco*RI (Gerlich, 1993). The L strand is complete apart from a nick of one or a few nucleotides at a fixed position about 1800 nucleotides from the *Eco*RI site while the S strand is incomplete (lacking 15-50% of the nucleotides), thereby producing a characteristic gap in the genome. The 5' end of the positive strand is located at a fixed point approximately 1560 nucleotides from the *Eco*RI site (Siddiqui et al. 1979). The fixed 5' ends of the two strands overlap by about 250 to 300 base pairs and comprise the so-called cohesive end region(Sattler and Robinson, 1979) which maintains the circular shape of the genome and prevents the two strands falling apart. The HBV genome is remarkably compact (Fig. 1.2). There are six overlapping open reading frames (ORFs), four of which are known to code for viral polypeptides: S (surface), C (nucleocapsid or core), P (polymerase) and X. The S ORF codes for three envelope polypeptides, designated "large" (LHBs), "medium" (MHBs) and "small" (SHBs) according to length (Heermann et al. 1984). The SHBs is the most abundant and contains the group "*a*" and subtype "*d/y*" and "*w/r*" determinants. It is now known that the subtypes arise through single amino acid substitutions in the polypeptides (Heermann et al. 1984; Ohnuma et al. 1993). However, these subtypes may occur in different strains of HBV, at least six genotypes (A-F) of which have been identified (Heermann et al. 1984; Norder et al. 1992).

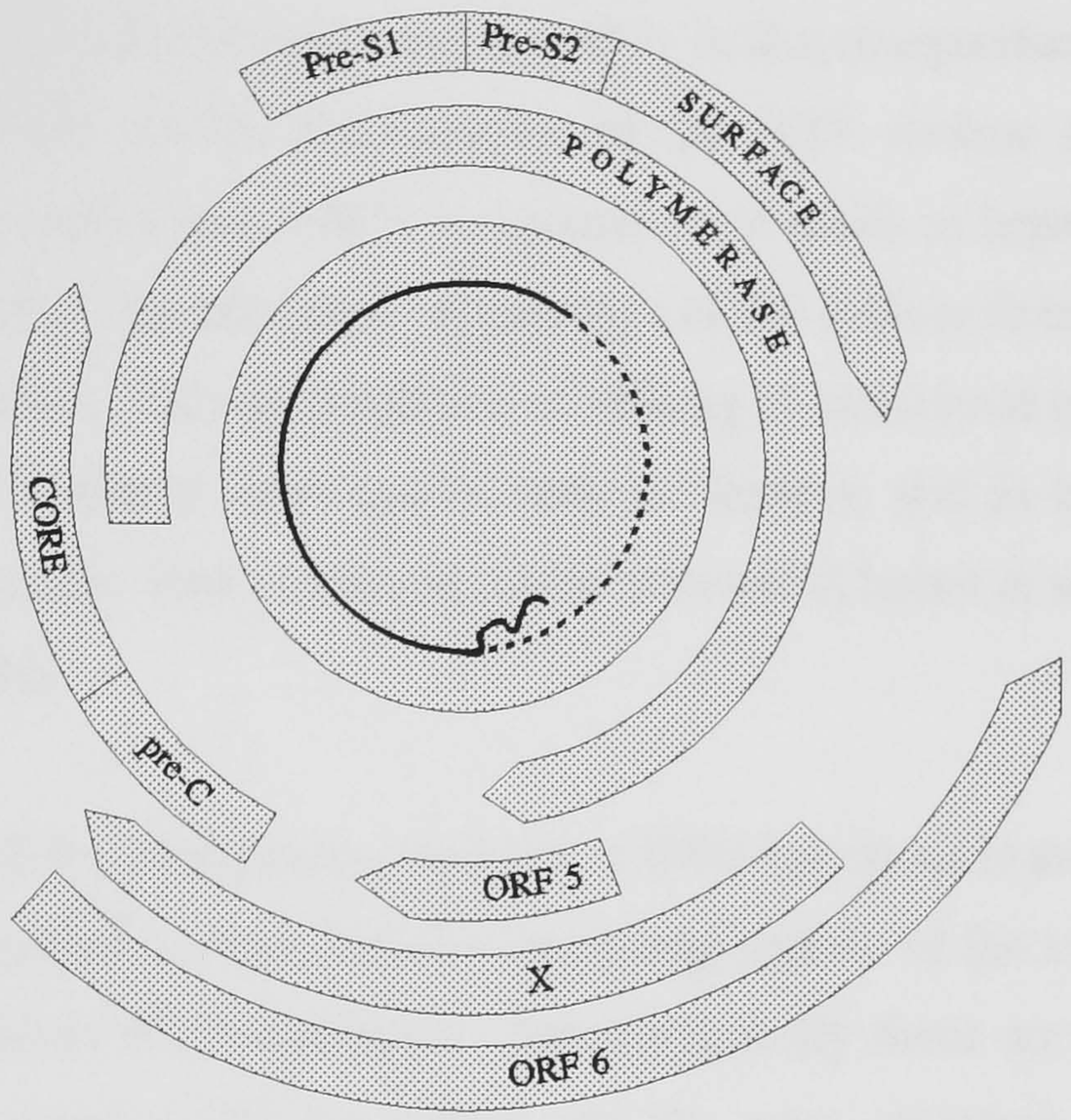


Figure 1.2 Schematic organization of the hepatitis B virus genome.

The MHBs comprises the SHBs with a 55 amino acid N-terminal extension containing the pre-S2 region. The importance of the pre-S2 region relates to its capability for binding aggregated serum proteins, notably polyaggregated human serum albumin (pHSA). It was suggested that, through binding to pHSA receptors on liver cells, this might provide a means for HBV to enter hepatocytes (Neurath et al. 1986) but this seems unlikely because pHSA is never found circulating in human subjects (Yu et al. 1985). Two other possible mechanisms of attachment of HBV to hepatocytes involving the pre-S2 region have been proposed. Since the SHBs is glycosylated (Heermann & Gerlich, 1991), binding via glycan receptors on liver cells might occur. Alternatively, binding may be via the amino-terminal portion of pre-S2 to transferrin receptors(Franco et al. 1992). However, there is still insufficient concrete evidence to support any of these mechanisms as an explanation for the hepatotropism of HBV (Gerlich, 1993).

The LHBs comprises the complete polypeptide encoded by the S ORF, including the pre-S1 domain. There is some evidence that pre-S1 may be involved in anchoring to cell membranes (Persing et al. 1987) and that pre-S1 sequences are involved in the binding of virions to the IgA receptor on the surfaces of hepatocytes, which may be the initial step in the infectious cycle (Neurath and Strick, 1990; Pontisso et al. 1992). Pre-S1 also exerts important structural effects on viral particle formation and secretion, in that overproduction of the LHBs relative to MHBs and SHBs inhibits the secretion of the HBV surface antigen (HBsAg) (Standring et al. 1986; Ou and Rutter, 1987), accumulation of which in hepatocytes probably accounts for the characteristic "ground glass" appearance of the cells in liver sections stained with haematoxylin and eosin. This accumulation of HBsAg is considered typical of chronic hepatitis B, and there is evidence from studies both in humans and in transgenic mouse lineages that it may eventually lead to hepatocellular necrosis (Chisari et al. 1987; Chisari, 1989; Dunsford et al. 1990).

In common with all hepadnaviruses, viraemia in HBV infections is associated with the copious production of incomplete viral particles consisting mainly of the virus coat protein (HBsAg), which are shed into the bloodstream. Morphologically these are of two principal types: small spheres of about 17-25 nm, which are the most abundant, and tubular or filamentous structures of about 20 nm diameter which vary in length. The available evidence suggests that the morphology of these particles is determined by the relative proportions of SHBs, MHBs and LHBs they contain (Marquardt et al. 1987).

The C ORF encodes two polypeptides (core and pre-core) that are identical except for 29 amino acids at the N-terminal end of the longer pre-core polypeptide(Miller et al. 1989). The core polypeptide is a nucleic acid binding component which encapsulates the viral nucleic acid [(Petit and Pillot, 1985). The pre-core polypeptide is not required for viral replication but its proteolytic cleavage products, so-called "e" antigen (HBe), are secreted into the blood and their presence is usually indicative of active viral replication (Standring et al. 1988). The function of HBe is unclear but it may act to confuse or suppress host immune responses to HBV infected cells. Seroconversion with development of anti-HBe antibodies (see below) and disappearance of HBe is usually associated with cessation of active viral replication. However, the importance of pre-core in the transport and formation of serum HBe has been emphasised by the finding of an HBV mutant occurring in patients with anti-HBe antibodies

who are also seropositive for HBV-DNA (indicating ongoing viral replication) and have severe active liver disease(Carman et al. 1992; Brunetto et al. 1991; Brunetto et al. 1990). This mutant has been characterised as having a single point mutation in the pre-core region which generates a translational stop codon. As a consequence, HBe production is abrogated and this explains the persistence of serum HBV-DNA in the presence of anti-HBe(Akahane et al. 1990; Tong et al. 1990).

The P ORF overlaps all of the other ORFs. The full functions of all of the products encoded by the P ORF are not yet known but include the viral polymerase/reverse transcriptase activity and primase activity with potential for generating minus strand synthesis(Mack et al. 1988; Schlicht, 1991). The X ORF comprises a short gene that partially overlaps the core gene and codes for a protein of 154 amino acid residues, the *in vivo* function of which is still obscure but which seems to exhibit gene transactivator properties and tumourigenic activity (Gerlich, 1993). Evidence that HBxAg with trans-activating properties can be made from a viral template integrated into the host genome during chronic infection (Wollersheim et al. 1988) supports the hypothesis that HBxAg may play an important role in the pathogenesis of chronic infection (Wang et al. 1991).

By analogy with other hepadnaviruses, it is thought that the HBV genome contains two other ORFs: ORF5 and ORF6 (Wang et al. 1991; Miller, 1988; Kaneko and Miller, 1988). ORF5 ranges from 70-100 codons in size and overlaps the P and X ORFs. It is uncertain how the gene sequence of ORF5 is translated because it lacks a standard in-phase AUG intiation codon. However, its 5' end contains an AUG codon which is believed to initiate protein synthesis in a gene of the adenovirus-associated virus (Anderson and Buzash Pollert, 1985). Alternatively, it is possible that the ORF5 product might be expressed as a fusion polypeptide through ribosomal frame-shifting during translation of the X and/or P ORFs, or that the viral mRNA is spliced and an initiation codon is fused in-phase to the 5' end of ORF5. ORF6 is believed to be located on the viral plus strand and therefore cannot be expressed from any of the known viral transcripts but may be translated from an antisense mRNA. It may encode a 210 amino acid polypeptide that overlaps the P, X, pre-core and ORF5 gene sequences of the complementary minus strand. An in-phase AUG codon is present near the start of the ORF6. The status of ORF6 is still hypothetical but it is possible that it may be involved in bidirectional transcription of the HBV genome. Bidirectional transcription is a rare event but

is known to occur in viruses such as adenovirus, simian virus 40 and herpes virus, and the anti-sense or latency-associated transcript is known to be present only in latently infected neurons(Anderson and Buzash Pollert, 1985; Puga and Notkins, 1987; Rock et al. 1987; Wagner et al. 1988; Stevens et al. 1987; Spivack and Fraser, 1987). Thus it is possible that a latency-associated anti-sense transcript exists during a specific point of HBV replication which is translated into the ORF6 gene product, the role of which in the virus' life cycle remains obscure.

Replication begins with conversion of the partially double-stranded HBV-DNA to a fully double-stranded circle by HBV-DNA polymerase in the hepatocyte nucleus (Fig. 1.3). The L (-) strand is transcribed by host cellular RNA polymerase to give multiple copies of a pre-genomic 3.5 kb RNA, which are then transported to the cytoplasm. Here, the core protein is made and this encapsulates the pre-genomic RNA together with newly synthesised HBV-DNA polymerase and DNA-linked protein. A new L (-) strand is then synthesised by reverse transcription of the pre-genomic RNA, during which the latter is degraded except for a small fragment which is thought to provide a primer for the synthesis of a new S (+) strand from the newly formed L (-) strand template. The progeny particles are thought to acquire HBsAg-containing envelopes by budding from the cell's plasma membrane, thereby becoming infectious particles.

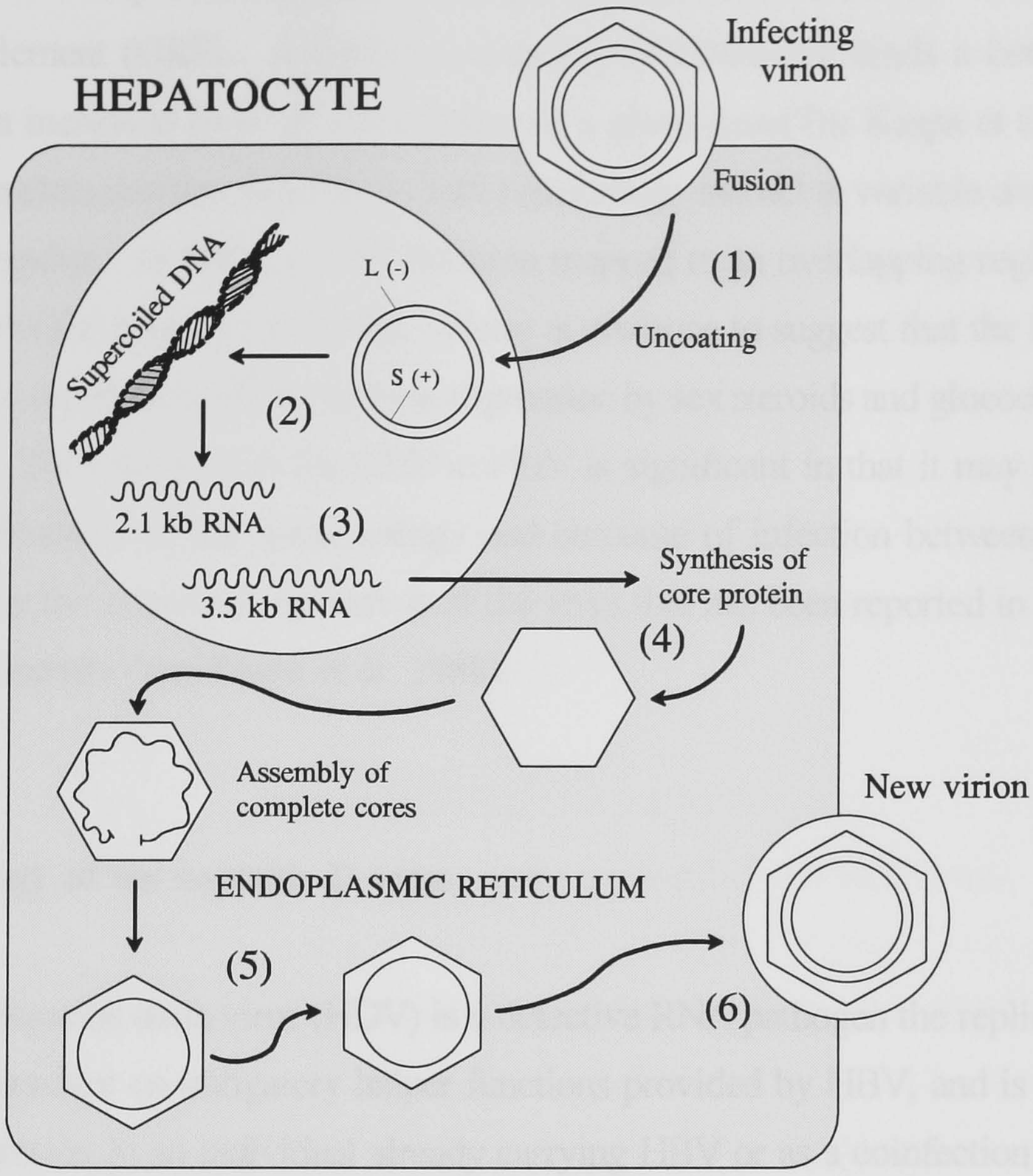


Figure 1.3 **Diagrammatic representation of events occurring during replication of hepatitis B virus in hepatocytes.** (1) The virus penetrates the hepatocyte, via a specific receptor on the cell membrane which recognises a peptide encoded by pre-S1, and uncoats. (2) In the nucleus, the partially double-stranded HBV-DNA is converted by DNA polymerase into a fully double-stranded circle. (3) L(-) strand is transcribed by host cellular RNA polymerase to provide multiple copies of a 3.5 kb RNA (pre-genome). (4) The RNAs are transported to the cytoplasm where the core protein is made and encapsulates the pre-genome RNA and the newly synthesised DNA polymerase and DNA-linked protein. (5) L(-) strand is synthesised by reverse transcription of the pre-genome, which is concomitantly degraded except for a small fragment probably used to prime the synthesis of the S(+) strand from the newly-made L(-) strand template. (6) The progeny particles obtain HBsAg-containing envelopes, probably by budding from the cell's plasma membrane, and become infectious virions. *Re-drawn from* Bonino, (1992).

One other important feature of the HBV genome is the existence of a glucocorticoid-responsive element (GRE). A GRE is a segment of DNA that binds a hormone receptor, promoting an increased level of transcription of a given gene(Tur Kaspa et al. 1988) GREs are cis-acting elements that function in both orientations and act at variable distances from the genes they regulate. In HBV, a GRE has been mapped to an overlapping region between the P and S ORFs (Tur Kaspa et al. 1986). There is evidence to suggest that the HBV GRE may be involved in regulation of HBsAg gene expression by sex steroids and glucocorticoids (Farza et al. 1987). Thus the role of the GRE in HBV is significant in that it may account for the observed differences in the epidemiology and outcome of infection between the sexes (see below) and for the enhanced replication of the virus that has been reported in patients treated with corticosteroids (Tur Kaspa et al. 1988)

1.3.2 Biology of the hepatitis D virus

The hepatitis delta virus (HDV) is a defective RNA pathogen the replication of which is wholly dependent on obligatory helper functions provided by HBV, and is acquired either as a superinfection in an individual already carrying HBV or as a coinfection with the initial HBV inoculum (Taylor et al. 1987; Hoofnagle, 1989). HDV has still not been classified but it has some resemblance to the plant viriods and satellite viruses (Branch et al. 1990).

The molecular basis of the biologically intriguing association between HBV and HDV is still unclear. When HDV superinfection occurs in a chronic HBV carrier, the HBe status appears not to be a determining factor. Both HBe and anti-HBe positive individuals seem to have equal susceptibility for HDV infection and seroconversion from HBe to anti-HBe and diminished HBV replication occur as a result of HDV. On the other hand, replication of both viruses, evidenced by high levels of the HDV-specific antigen (HDAg) and HBc in serum and hepatocytes has been documented. Such cases are more common in drug addicts and are notable for the aggressive nature of the disease, which suggests a compound pathogenic effect of the two viruses (Kuo et al. 1989; Monjardino and Saldanha, 1990). It is not yet known how HDV contributes to the worsening of the underlying HBV hepatitis, nor how it induces acute fulminant hepatitis in a number of cases of co-infection with HBV(Negro and Rizzetto, 1993).

HDV is the smallest known human virus. The complete virion is a 36 nm particle containing the viral genome - a circular, single-stranded RNA 1.7 kb in length and of negative polarity (Wang et al. 1986; Makino et al. 1987; Saldanha et al. 1987). Although several open reading frames have been identified, only one (ORF5) has been demonstrated to encode a protein (HDAg). The viral RNA has long regions of complementarity that enables the opposite ends to bind to each other, resulting in a rod-like structure(Kos et al. 1986). The genomic RNA is copied in a rolling circle to give a chain of repeated antigenomic molecules. Each new circle is then used as a template to create many new copies of the original molecules. There is no evidence of the involvement of any enzymes in HDV replication. Rather, the cleavage and subsequent self-ligation of HDV seems to be a function of the genomic RNA itself(Sharmeen et al. 1989; Wu and Lai, 1989).

1.3.3 Epidemiology and outcome of hepatitis B and D virus infections

The hepatitis B and D viruses are both transmitted parenterally. HBV has a worldwide distribution. Overall, the frequencies of chronic infection are highest in the Far East, other parts of Asia, and in parts of Africa, intermediate in the Mediterranean area and South America, and generally low in northern Europe and North America. In the latter areas, however, the carriage rate is high in certain risk groups, particularly among promiscuous heterosexuals, homosexuals and intravenous drug users (Yoffe and Noonan, 1992). The large majority of chronic carriers of HBV elsewhere in the world have been infected perinatally. It is generally thought that only about 5% of adults infected with HBV become chronic carriers(McMahon et al. 1985) but a recent follow-up study of more than 55,000 U.S. army personnel infected through contaminated yellow fever vaccine in World War II suggests that the proportion may be much lower (Norman et al. 1993). However, males infected in adulthood are more likely to become carriers than females (Jacyna and Thomas, 1993), suggesting that host hormonal factors may have a role to play in presistence of the infection (see above, Section 1.3.1).

HDV also has a worlwide distribution but this is somewhat irregular and data are lacking from some areas. In particular, there are few data available on the prevalence of HDV in Africa. A high rate of HDV infection has been reported from the northern part of South

America. Studies in Colombia and among the Yucpa Indians in Venezuela, where it has been associated with outbreaks of severe hepatitis with a high prevalence of fulminant hepatitis, have shown that as many as 60-80% of HBsAg carriers are seropositive for anti-HDV antibodies (Jacyna and Thomas, 1993; Buitrago et al. 1986). Similarly high rates have been reported from Central Africa, Asiatic Russia, Romania and Kuwait (Tapalaga et al. 1987; Greenfield et al. 1986; al Kandari et al. 1988). On the other hand, in some areas where the HBV carriage rate is very high, such as in China, Japan, Southern Africa and among the Alaskan Eskimos, evidence of exposure to HDV is rarely found(Rizzetto et al. 1991). High rates of HDV infection among HBV carriers have been noted in southern Italy but elsewhere in the Mediterranean area lower frequencies of exposure to HDV are found (Rizzetto et al. 1991). In northern Europe and North America the frequency in the general population is low but, as with HBV, in these areas there are certain groups at particular risk. In contrast to HBV, however, the rate of HDV infection among homosexuals is relatively low (< 15%) but 30-75% of intravenous drug users and multiply-transfused HBsAg-positive haemophiliacs have evidence of HDV exposure (Rizzetto et al. 1988a; Rizzetto et al. 1980; Solomon et al. 1988; Pol et al. 1989; Smith et al. 1992). The reasons for this variability in HDV infection are not clear. The prevalence of intravenous drug use in different populations is clearly a factor, but sexual transmission also occurs(Wu et al. 1995).

The spectrum of liver disease in subjects chronically infected with HBV varies widely. Many carriers will remain "healthy", with normal biochemical liver tests and only minimal changes on liver biopsy, while others will have a severe periportal hepatitis with rapid progression to cirrhosis (Hoofnagle, 1989; Scheuer and Lefkowitch, 1994). Prognosis is related to the duration and severity of underlying liver disease but is unpredictable and depends also on whether there is concomitant infection with HDV or the hepatitis C virus (see below). Most patients with periportal hepatitis and cirrhosis remain relatively stable for many years before they decompensate, with development of ascites, jaundice, hepatic encephalopathy or variceal bleeding but, as noted above, there is also a risk for development of hepatocellular carcinoma (HCC) (Szmuness, 1978; Beasley et al. 1981; Kew and Popper, 1984). Whether HBV has inherent oncogenic potential is still uncertain. In most cases of HCC in chronic hepatitis B, the malignancy has developed against a background of long standing infection and cirrhosis. But cirrhosis itself is an independent risk factor for HCC (Kew and Popper, 1984; Zaman et al. 1985).

A recent study of 349 Western European patients with chronic hepatitis B and cirrhosis has shown that the cumulative probability of decompensation was 23% at 5 years and 37% at 10 years (Fattovich et al. 1995). Five year survival after the first major decompensation was only 35%. Advanced stage and duration of cirrhosis, and thrombocytopenia, were the only independent variables for development of HCC. Neither HBeAg/HBV-DNA seropositivity nor concomitant HDV infection were prognostic factors. Cumulative probability of developing HCC was 6% at 5 years and 15% at 10 years. It was concluded that: (1) patients with well compensated HBsAg-positive cirrhosis do not experience worsening of their condition for several years, but once decompensation occurs life expectancy is poor, (2) HDV does not appear to play a pathogenetic role in development of HCC, and (3) the findings support the concept that HCC development may be a consequence of long-standing cirrhosis.

1.3.4 Serology and diagnosis of hepatitis B and D virus infections

The serological events occurring after exposure to HBV define the status of the infected individual. The prototype marker is HBsAg. In uncomplicated acute hepatitis B, HBsAg may be detected in the blood shortly before the first rise in serum aminotransferases (Fig. 1.4). The latter is usually associated with development of the symptoms and clinical features of hepatitis including jaundice but, as noted above (Section 1.2.1), anicteric and even asymptomatic infections are not uncommon. At this stage also, the patient will usually be seropositive for HBeAg and HBV-DNA and will be highly infectious. IgM class antibodies (IgM anti-HBc) against the HBV core antigen also begin to appear at this time. Seroconversion from HBeAg to anti-HBe (with disappearance of HBV-DNA) occurs between a few weeks to several months after the initial acute phase. This coincides with a reduction in viral replication in the liver and resolution of hepatic inflammation, and with the development of a secondary (IgG) antibody response to HBc, and often also of IgA anti-HBc (Nomura et al, 1985). IgM and IgA anti-HBc, along with anti-HBe, gradually disappear during the recovery phase, followed by the appearance of antibodies (anti-HBs) against HBsAg. The latter also declines in titre over the succeeding months and may eventually become undetectable, but IgG anti-HBc usually persists for life and, on its own, is considered to the be best indicator of past (completely resolved) infection.

Failure to clear HBsAg within six months defines a chronic HBV carrier state. Such individuals are usually also HBeAg seropositive. IgG anti-HBc very often appears but IgM anti-HBc production continues for a time - at reducing titres (Sjogren and Hoofnagle, 1985; Hoofnagle et al. 1987; Kiyosawa et al. 1988). Persistence of HBsAg and HBeAg without development of anti-HBe or anti-HBs may last for life but the majority of patients will seroconvert to anti-HBe at some stage (six months to several years after infection), remaining seropositive only for HBsAg and anti-HBc. Seroconversion is often accompanied by what, clinically and biochemically, appears to be a second episode of acute hepatitis which, in some cases, may be due to delta virus (HDV) superinfection (see below).

Although these two general patterns of serological events are seen in the majority of patients with either uncomplicated acute or chronic HBV infection, recent evidence indicates that they are not invariable. For example, it is possible for individuals to clear HBsAg and become anti-HBs seropositive while still harbouring the virus in their livers and secreting small amounts of HBV-DNA into the blood (Chung et al. 1995). The pathogenic significance of such small amounts of virus is currently unclear but these individuals may continue to be potentially infectious. This situation is thought to arise because of the development of "escape" mutants of HBV. In addition to the pre-core mutation discussed above (Section 1.3.1), several other mutations have been described which are presumed to develop under selective pressure of the host immune system (Mimms, 1995; Kato et al. 1996). These are often associated with clearance of HBsAg and/or HBeAg and seroconversion to anti-HBs and/or anti-HBe, and continuing active viral infection can usually be determined serologically only by detection of circulating HBV-DNA.

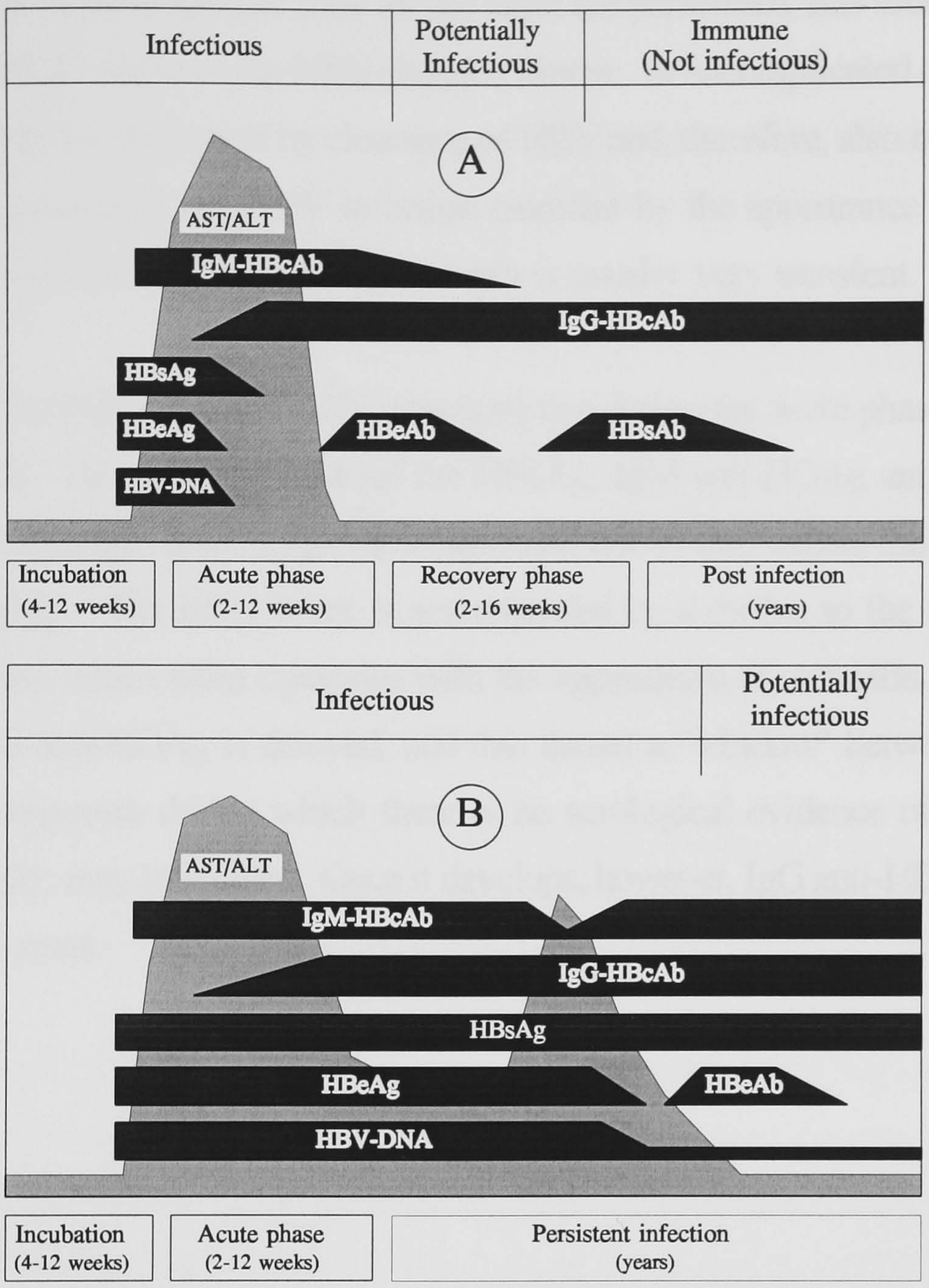


Figure 1.4 **Diagrammatic representation of typical serological events occurring during uncomplicated acute (Panel A) and chronic (Panel B) hepatitis B virus infection.** For details see text. *Adapted from Hoofnagle and Di Bisceglie, (1991).*

The serology of HBV is further complicated if there is concomitant infection with HDV. The sequence of events depends to some extent on when the HDV infection is acquired and the host's response to it. Because of its close dependence on, and inter-relationship with HBV, it is customary to measure and interpret serological markers of HDV infection in conjunction with HBV markers. Indeed, HDV interferes with HBV replication and can thus have profound effects on HBV serum markers (Di Bisceglie, 1993; Rizzetto et al.

1988b). *Co-infection* with HDV in the initial HBV inoculum can lead to a biphasic acute hepatitis with a periodicity of up to six weeks in which the first episode is due to HBV and the second to HDV. Unless specific tests for the latter are performed, this second episode may be misinterpreted as a relapse of the HBV-induced illness. In uncomplicated cases, the second episode is commonly accompanied by clearance of HBV and, therefore, also of HDV. In acute hepatitis following *co-infection*, HDV infection manifest by the appearance of HDAg in the blood is rarely demonstrable because the viraemia is usually very transient.

HBsAg titres (reflecting the HBV infection) rise during the acute phase and fall during recovery (Fig. 1.5). At about the peak of the HBsAg, IgM anti-HDAg antibodies begin to appear (together with high titres of IgM anti-HBc) and rise in titre during the late acute phase before falling rapidly. The latter event is accompanied by a switch to the secondary (IgG) anti-HDAg response which often coincides with the appearance of anti-HBs. Sometimes the appearance of IgG anti-HDAg is delayed, and this leaves a "window" between the IgM and IgG anti-HDAg responses during which there is no serological evidence of HDV infection which, consequently, may be missed. Once it develops, however, IgG anti-HDAg usually then persists for many years.

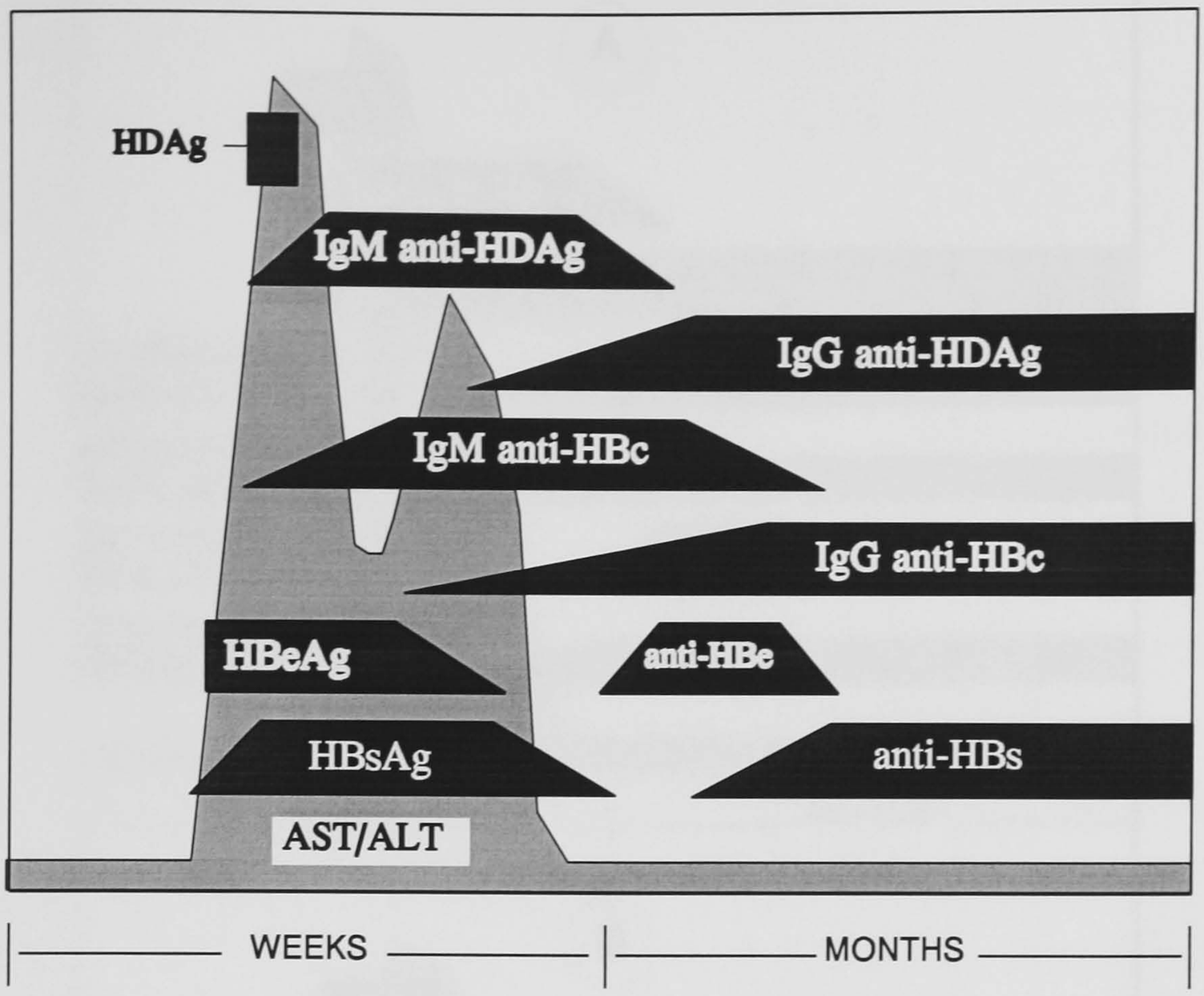


Figure 1.5 **Diagrammatic representation of the typical serological events associated with acute hepatitis B (HBV) and delta virus (HDV) *co-infections*.** For details see text. *Adapted from Hoofnagle and Di Bisceglie (1991).*

When HDV is acquired as a *super-infection* in a chronic HBV carrier, the serological pattern of events is somewhat different. HDAg usually appears in the serum at the onset of the acute phase of the HDV-induced hepatitis, together with IgM anti-HDAg (Fig. 1.6). This is often preceded by a decline in all of the markers of the underlying HBV infection, due to suppression of HBV replication by HDV. The patient may become seronegative for IgM anti-HBc or have only very low titres and, in HBeAg-positive individuals, there is almost always seroconversion to anti-HBe. Thereafter, the sequence of events depends on the clinical outcome. In uncomplicated acute HDV hepatitis, the viraemia will wane quite rapidly, IgM anti-HDAg will usually disappear within a few months after recovery, and persistence of IgG anti-HDAg will be the only lasting evidence of the HDV infection. However, the majority of super-infected individuals seem to develop chronic HDV infections (Rizzetto et al. 1988a; Dimitrakakis et al. 1986), with persistence of both IgM- and IgG-anti-HDAg, and often with on-going liver damage - whether or not there is biochemical evidence of liver dysfunction. Such individuals often also have circulating IgA anti-HDAg antibodies, which seem to be particularly associated with active liver necroinflammation (McFarlane et al. 1991).

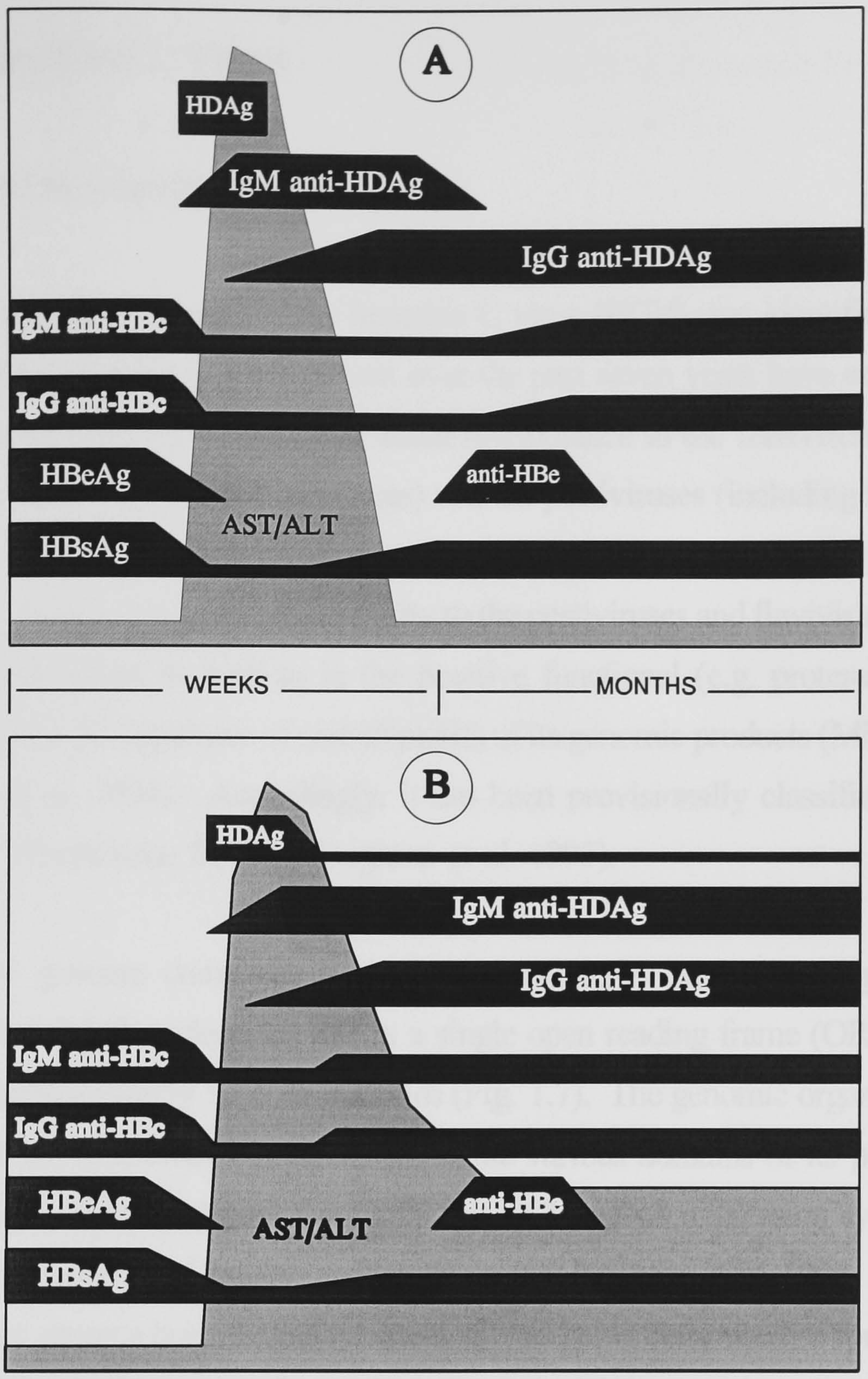


Figure 1.6 **Diagrammatic representation of the typical serological events associated with acute (Panel A) and chronic (Panel B) hepatitis delta virus (HDV) *super-infection* in a chronic hepatitis B carrier.** For details see text. *Adapted from Hoofnagle and Di Bisceglie (1991).*

1.4 THE HEPATITIS C VIRUS

1.4.1 Biology of the hepatitis C virus

As noted above (Section 1.2) the hepatitis C virus (HCV) was identified by Choo and colleagues in 1989. Intensive investigations over the past seven years have established that it is a small enveloped RNA virus that bears some resemblance to the flaviviruses (e.g. dengue, yellow fever and Japanese encephalitis viruses) and the pestiviruses (including hog cholera and bovine diarrhoea viruses). While there is little primary genomic sequence identity between HCV and these viruses, there is considerable similarity to the pestiviruses and flaviviruses with respect to its overall organisation, as well as in the putative functional (e.g. protease and helicase) activities and amino acid sequences of certain motifs of its genomic products (Miller and Purcell, 1990; Houghton et al. 1994). Accordingly, it has been provisionally classified as a separate genus within the *Flaviviridae* family (Houghton et al. 1993).

The HCV genome comprises a single-stranded RNA molecule of positive (sense) polarity, with about 9,500 nucleotides (nt) in a single open reading frame (ORF) that encodes a polyprotein of approximately 3000 amino acids (Fig. 1.7). The genomic organisation of HCV and the structure and functional characteristics of the various domains of its polyprotein have been deduced largely from analyses of recombinant HCV cDNA expression systems and have been extensively reviewed by Houghton and colleagues (Houghton et al. 1993; Houghton et al. 1994). Briefly, the genome is envisaged schematically as being comprised of four domains: two untranslated regions (UTRs) at the 5' and 3' ends of the RNA strand, and a structural and a non-structural region (Fig. 1.7). The 5' leader sequence of 341 nt is highly conserved and has a complex secondary organisation involving several stem-loop structures which are thought to be important for genomic translation and replication and assembly of the virus. The 3' UTR is very short (27-45 nt) and is more variable, but seems to contain some conserved sequences and stem-loop secondary structures that may be important in the virus' replication. The structural domain encodes three proteins: a capsid (or "core") protein (C) of 21-26 kilodaltons (kD) with a basic N-terminal segment and two glycosylated proteins, designated E1 or gp33 (31-35 kD) and E2 or gp72 (68-72 kD), which are thought to constitute the viral envelope. Like the 5' UTR, the core region is highly conserved. In contrast, the E2 region is hypervariable, and recent evidence suggests that rapid amino acid substitutions in the products of this region may be a

mechanism by which the virus adapts for persistent infection (Kurosaki et al. 1993; Bonino et al. 1993; Yamaguchi et al. 1994). In any event, this variability has major implications for the development of vaccines against HCV.

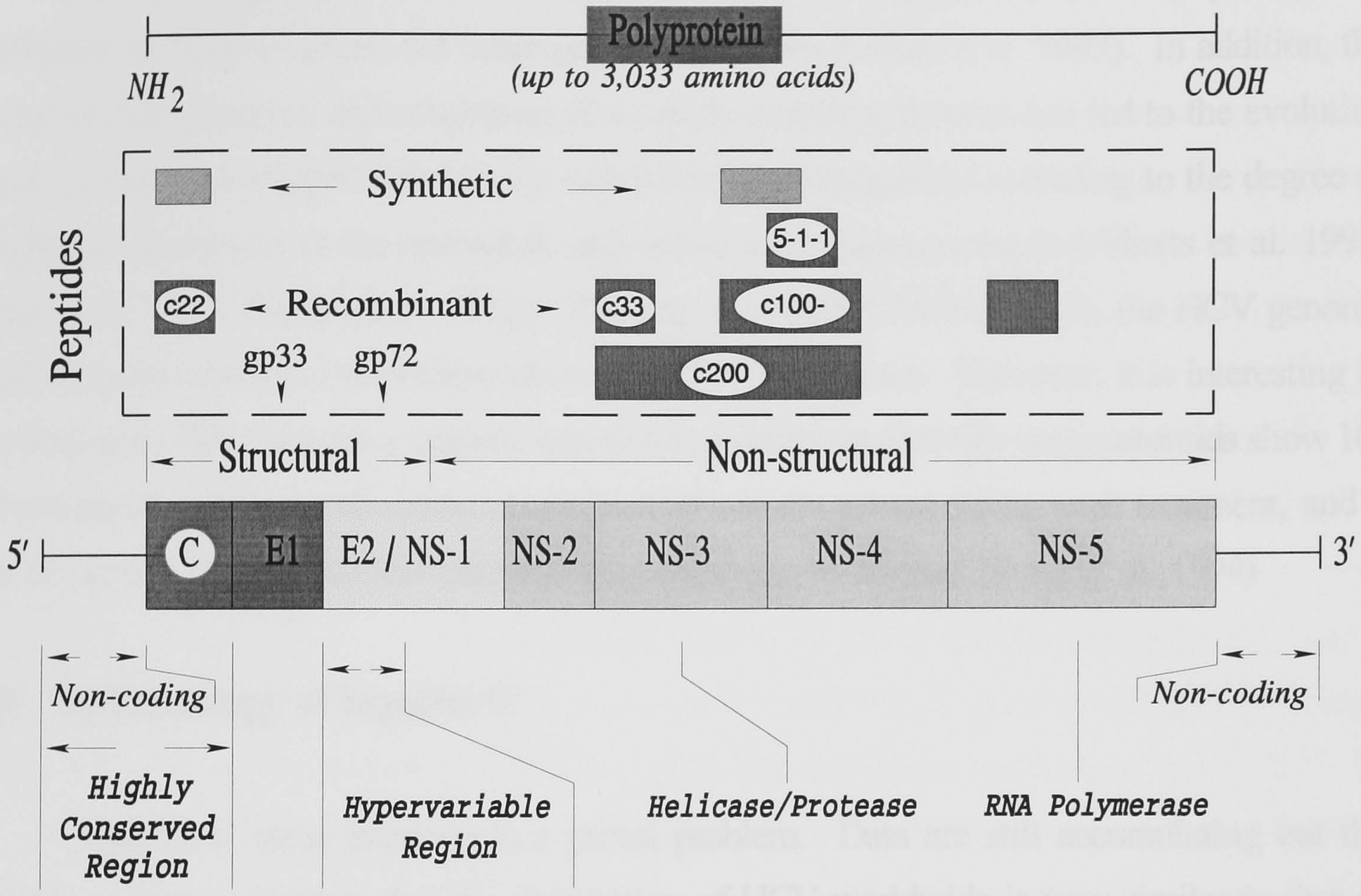


Figure 1.7 **Postulated genomic organisation of the hepatitis C virus.**
Adapted from Houghton et al (1994).

The products of the non-structural domain are believed to be involved in replication of the virus and in cleavage of the transcribed polyprotein. The functions of the products of these regions (NS1-NS5) appear to include various proteinase and helicase activities. The large NS5 region is thought to encode a number of peptides with multiple functions which, by analogy with other RNA viruses, probably includes a viral RNA-dependent polymerase.

The HCV genome is not a homogeneous species. Rather, it exists as a quasispecies distribution of closely related but heterogeneous genomes (Caban et al. 1993). In addition, the process of host selection and adaptation of a rapidly mutating genome has led to the evolution of many distinct genotypes (see below), which can be distinguished according to the degree of relatedness/divergence at the nucleotide and amino acid sequence levels (Alberts et al. 1991; Hadler et al. 1991; Ricchi et al. 1992). Whether, like HBV (Section 1.3.1), the HCV genome includes a glucocorticoid responsive element (GRE) is unknown. However, it is interesting to note that, as in HBV infection, chronic hepatitis C patients treated with corticosteroids show 10-fold increases viral genomic material (HCV-RNA) in the serum during such treatment, and a flare of serum aminotransferase activity when treatment is stopped (Fong et al. 1994)

1.4.2 Epidemiology of hepatitis C

Hepatitis C virus infection is a global problem. Data are still accumulating but the available evidence suggests that the distribution of HCV worldwide is very similar to that of HBV, i.e. relatively low prevalence in the temperate zones of northern Europe and North America, intermediate in the Mediterranean area, and high in Asia, Africa, parts of the Pacific basin and probably also at least in parts of Latin America (Van der Poel, 1994). There are, however, important differences from HBV in the risk factors for acquisition and the outcome of HCV infection.

It seems probable that, like HBV, HCV is transmitted only via the parenteral route. Nonetheless, in 30-80% of cases of chronic HCV infection there is no apparent history of a direct parenteral contact (Bonino et al. 1993). Careful anamnesis may reveal previously unsuspected parenteral exposure to blood or blood products in many cases (Chiaramonte et al. 1996) but, in others, the mode of acquisition in these so-called "community acquired" or "sporadic" infections remains an enigma. Studies of sexual and other modes of close-contact transmission of HCV have yielded conflicting results. Although most studies document a higher

rate of evidence of exposure to HCV in households where there is at least one infected member than in the general population or households in which there are no index cases, the incidence is relatively low (about 5% overall) and varies widely (from 0 to 30%) (Alter, 1994). This may be related to the status of the index case, for Buscarini et al [1993] found evidence of HCV exposure in 14.9% of members of families where one member had active liver disease but none in families where the index cases had normal serum aminotransferases. Shedding of HCV in saliva has been reported (Couzigou et al. 1993; Wang et al. 1992) but, in one of these studies (Wang et al. 1992), there was no evidence of transmission to spouses. Other studies (Hsu et al. 1991; Fried et al. 1992) found no evidence of the virus in the semen or saliva of infected individuals and concluded that there was no direct evidence of transmission by these routes. This is supported by other findings of a low incidence of HCV transmission in homosexual populations or between heterosexual partners (Everhart et al. 1990; Melbye et al. 1990; Kolho et al. 1991; Shev et al. 1991; Chiaramonte et al. 1996).

Data relating to vertical (maternal/foetal) transmission of HCV are also conflicting. A high rate of transmission to newborn children of mothers with human immunodeficiency virus (HIV) and HCV infections has been reported (Novati et al. 1992) and this is supported by other studies showing a high rate of HCV transmission between sexual partners in the HIV setting (Eyster et al. 1991), suggesting that HIV may modulate expression and infectivity of HCV. In the absence of HIV, the reported frequencies of maternal/foetal HCV transmission vary widely. Wejstål et al [1992] found that 18 of 21 children born to 14 women with chronic hepatitis C were transiently seropositive for anti-HCV antibodies during the first 3 to 12 months after birth and two had very transient viraemia, but concluded that these were probably cases of passive transfer of the virus and/or antiviral antibodies. Only one (4.8%) of the children developed a sustained viraemia. A very similar frequency (5.6%) was reported by Ohto et al [1994] in Japan, who noted that transmission to infants occurred only from mothers with high serum titres of HCV-RNA. In contrast, another Japanese study has reported a maternal/foetal transmission frequency of 33% (Kuroki et al. 1993). However, given the opportunity for direct blood-blood contact during the birth process, and in marked contrast to HBV, the overall picture suggests that the risk of maternal/foetal transmission of HCV is relatively low.

Also in marked contrast to HBV, up to 90% of adults infected with HCV become chronic carriers of this virus (Di Bisceglie et al. 1991; Yano et al. 1993; Cordoba et al. 1994). The frequency with which this occurs may not always be appreciated because of the clinically mild,

indolent and fluctuating course usually associated with chronic HCV infection. The large majority of such individuals are clinically asymptomatic and often have normal serum aminotransferases, even in the presence of severe underlying liver disease (Di Bisceglie et al. 1991; Alberti et al. 1992; Romeo et al. 1993; Shindo et al. 1995; Silini et al. 1995). It is possible that a true "healthy" carrier state exists (Prieto et al. 1995) but, while some chronically infected individuals have minimal or no histological changes on liver biopsy (Esteban et al. 1991), it seems that in most cases the disease slowly and inexorably progresses to cirrhosis (Cordoba et al. 1994). The reasons why there are differences between patients in the course of the disease are unknown but may be related to the level of viraemia which, in turn may depend on the particular genotype with which the individual is infected (see below). The mode of acquisition of infection may be another factor. Gordon et al. (1993) have reported that patients acquiring the virus through blood transfusion (and therefore presumably receiving a large inoculum) have more aggressive liver disease than HCV-infected intravenous drug users (who presumably receive smaller doses of the virus).

There is also increasing evidence that (like HBV) HCV is associated with development of hepatocellular carcinoma (HCC), particularly in Japan where up to 70% of HCC cases have evidence of HCV infection (Saito et al. 1990; Yuki et al. 1992; Shiratori et al. 1995), but also in areas of relatively low HCV endemicity (Paterlini et al. 1993). Nonetheless, in almost all cases the malignancy develops against a background of cirrhosis which, as noted above (Section 1.3.3), is itself an independent risk factor for HCC (Kew and Popper, 1984; Zaman et al. 1985; Okuda, 1992). Perhaps the most persuasive evidence has come from a recent retrospective study of patients with autoimmune hepatitis (AIH), in which HCC is normally very rare, showing that 75% of patients with AIH who developed HCC had previously undiagnosed superimposed chronic HCV infections (Ryder et al. 1995).

1.4.3 Variants of the hepatitis C virus

On the basis of nucleotide sequence divergence of up to 10% within, and of more than 20% between, HCV-RNA isolates from different countries, it has been established that there are a number of genotypes and subtypes of the virus (Simmonds, 1994). By comparison with the prototype virus (HCV-1, now more usually referred to as HCV-PT) early studies at Chiron Corporation initially led to classification of these variants into three groups: HCV-I, HCV-II and

HCV-III (Choo et al. 1991; Houghton et al. 1991) From studies of isolates from Japanese patients, Enomoto et al. (1990) had proposed an alternative classification: HCV-PT, -K1, -K2a and -K2b. Okamoto et al. (1992b) also identified four corresponding genotypes in Japan but suggested that they should be designated genotypes I, II, III and IV. Since then, a considerable amount of nucleotide sequence data has been obtained by different investigators and this has led to propose the classification shown in Table 1.1, which has been used throughout the present study (Simmonds, 1995; Mori et al. 1992).

There are distinct variations in the prevalence of the different genotypes in different geographical areas. Data are still accumulating but the available evidence, recently reviewed by Simmonds(Simmonds, 1995; Simmonds, 1994), suggests that genotypes 1 and 2 predominate in northern and southern Europe, North America and Japan. Type 3, which is rare in Japan and other countries along the north-western rim of the Pacific basin, accounts for the majority of infections in Thailand, Bangla Desh and India, while type 4 appears to be concentrated in the Middle East and in Central Africa (Simmonds, 1995). Types 5 and 6 seem to show a highly restricted distribution, the former having been reported mainly from South Africa and the latter from Hong Kong and Vietnam. Other, newer genotypes have been recently reported from Vietnam (Tokita et al. 1994).

Table 1.1 Proposed nomenclature of HCV genotypes compared with previous assignations

Genotype	Chiron	Enomoto	Mori/Okamoto
1a	I	K-PT	I
1b	II	K-1	II
1c	nc	nc	nc
2a	III	K-2a	III
2b	III	K-2b	IV
2c	III	nc	nc
3a	IV	nc	V
3b	IV	nc	VI
4	nc	nc	nc
5	V	nc	nc
6	nc	nc	nc

Adapted from Simmonds,(1995). nc = not classified. For other details see text.

In attempts to clarify the relationships between the different genotypes and subtypes, Simmonds and colleagues have undertaken detailed phylogenetic analyses of the nucleotide sequence data from HCV isolates from different parts of the world (Simmonds, 1995), as shown in the phylogenetic tree in Fig. 1.8, where branch lengths are proportional to the proposed evolutionary distances from each other and from a putative ancestral virus.

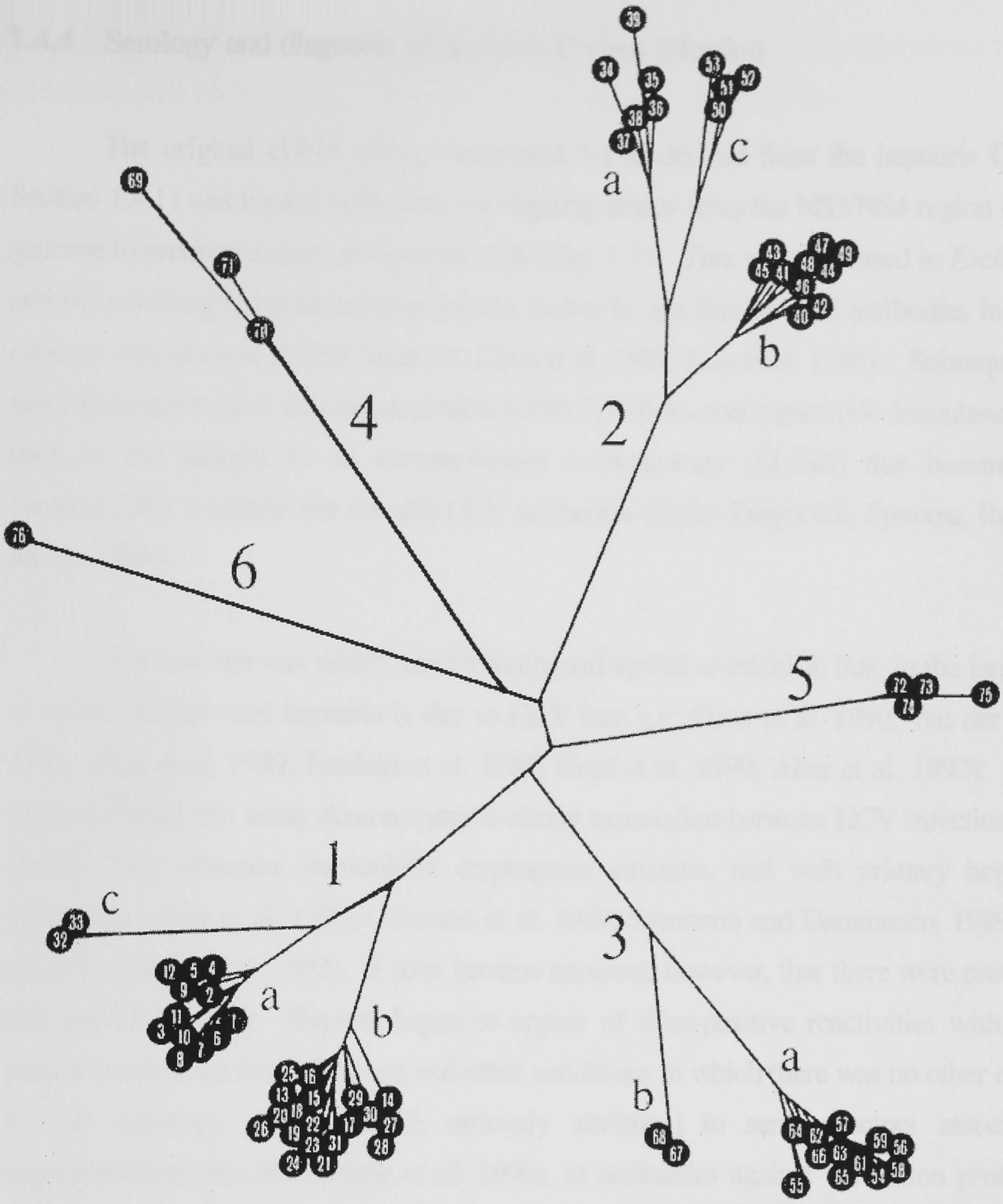


Figure 1.8 **Phylogenetic analysis of nucleotide sequences in part of the HCV NS-5 region.** *Re-drawn from (Simmonds, 1995).*

1.4.4 Serology and diagnosis of hepatitis C virus infection

The original cDNA clone, designated 5-1-1, derived from the hepatitis C virus (see Section 1.4.1) was ligated with three overlapping clones from the NS3/NS4 region of the HCV genome to produce a clone designated c100 (Fig. 1.7). This was expressed in *Escherichia coli* and the resulting recombinant protein was shown to specifically bind antibodies in the sera of patients with chronic NANB hepatitis (Choo et al. 1989; Kuo et al. 1989). Subsequently, c100 was expressed in yeast as a fusion protein (c100-3) with human superoxide dismutase (SOD) and used as the antigen in an enzyme-linked immunoassay (ELISA) that became the first commercially available test for anti-HCV antibodies (Ortho Diagnostic Systems, Raritan, New Jersey, USA).

The new test was widely used initially and served to establish that, in the large majority of cases, NANB viral hepatitis is due to HCV [see e.g. Choo et al. 1990; van der Poel et al. 1989; Alter et al. 1989; Esteban et al. 1989; Hopf et al. 1990; Alter et al. 1992). Other data obtained using this assay demonstrated a strong association between HCV infection and some chronic liver diseases, particularly cryptogenic cirrhosis, and with primary hepatocellular carcinoma (Alter et al. 1992; Colombo et al. 1989; Sansonno and Dammacco, 1989; Hasan et al. 1990; Jeffers et al. 1992). It soon became apparent, however, that there were problems with this anti-HCV assay. Reports began to appear of false-positive reactivities with sera from patients with some liver disorders and other conditions in which there was no other evidence of a viral aetiology. These were variously attributed to serum factors associated with hyperglobulinaemia (McFarlane et al. 1990), to antibodies against the fusion protein (SOD) (Ikeda et al. 1990), or to rheumatoid factor (Theilmann et al. 1990). Reactions against contaminating traces of yeast proteins in the recombinant c100-3 preparation that might have escaped the purification process also provided potential for false reactivity.

In addition, further studies revealed that the appearance of anti-c100-3 antibodies is unpredictable. Cuypers et al. [1991] found that these antibodies often appeared relatively late (and sometimes not at all) in cases of transfusion-related HCV infection, whereas patients frequently had antibodies against additional epitopes that had been identified in peptides derived from the nucleocapsid (c22) and NS3 (c33) regions (Van der Poel et al. 1991). However, the patterns of response to these additional peptides also varied between individuals (Fig. 1.9). Since several of these different patterns were observed in recipients infected by blood products

from a single donor, (Cuypers et al. 1991) concluded that they were determined by the host response to the virus rather than to differences between virus inocula.

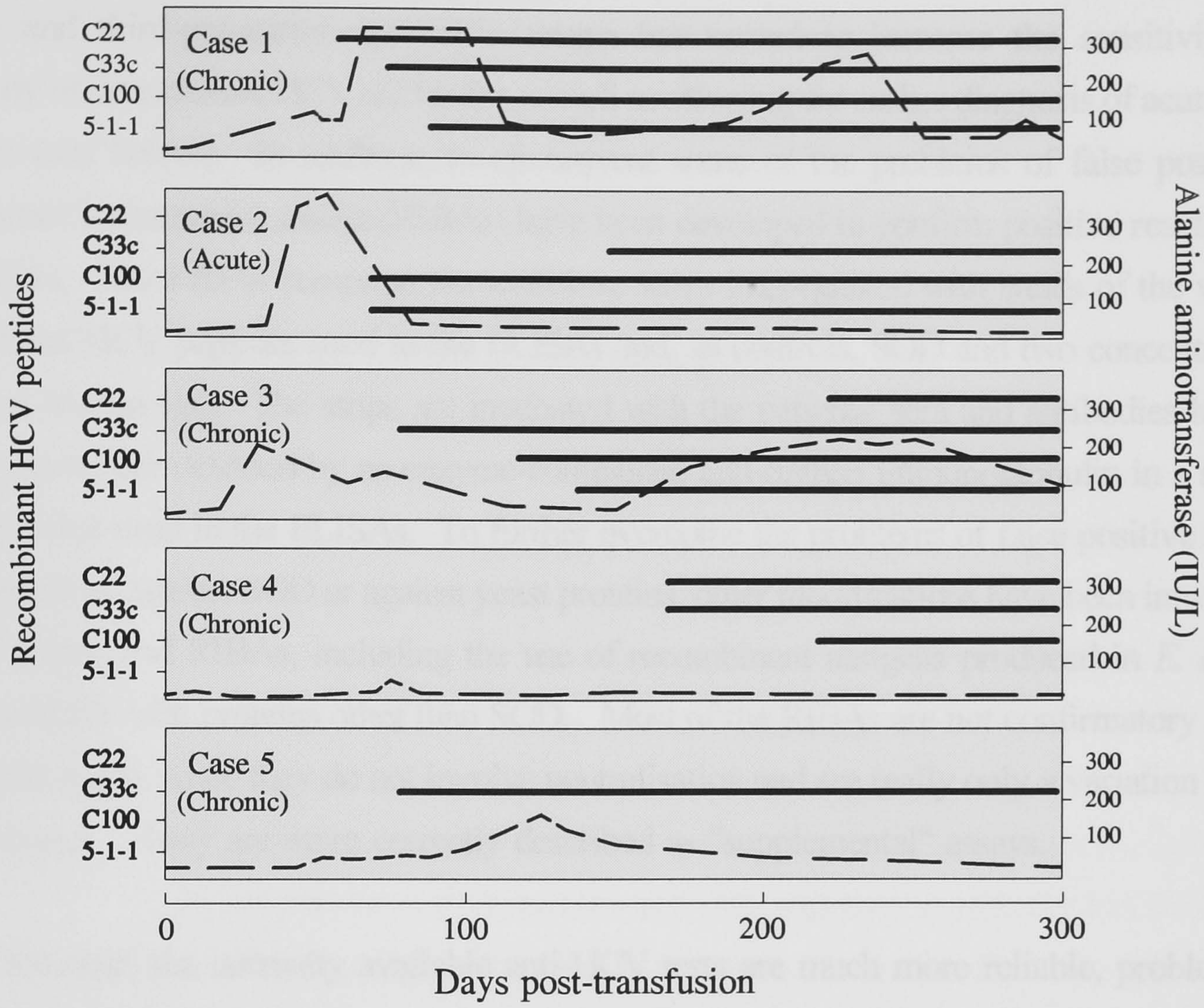


Figure 1.9 **Antibody responses to different HCV peptides in post-transfusion HCV infection.** Solid horizontal bars represent seropositivity for antibodies against the different peptides shown on the left. Broken line = alanine aminotransferase (ALT) activities. Chronic infection was determined by seropositivity for HCV-RNA at various times after 250 days post-transfusion. *Adapted from* (Cuypers et al. 1991).

As useful as they have been, the early assays based only on c100-3 are now virtually obsolete and new assays for HCV antibodies have been developed that incorporate additional epitopes in recombinant (Choo et al. 1990) or synthetic (Hosein et al. 1991) HCV peptides derived from the nucleocapsid and from the NS3 and NS4(Alter et al. 1992) regions and, more recently, a peptide from the NS5 region(Courouce and Janot, 1994). The introduction of these second- and third-generation anti-HCV assays has served to increase the sensitivity and specificity of detection of HCV antibodies as well as allowing for earlier diagnosis of acute HCV infection (see below). In addition, to circumvent some of the problems of false positivity, recombinant immunoblot assays (RIBAs) have been developed to confirm positive reactions in the ELISAs. The RIBAs comprise nitrocellulose strips impregnated with bands of the various recombinant HCV peptides used in the ELISAs and, as controls, SOD and two concentrations of normal human IgG. The strips are incubated with the patients' sera and antibodies binding to the peptides are detected by an enzyme-conjugated anti-human immunoglobulin in a similar manner to that used in the ELISAs. To further overcome the problems of false positive results due to reactions against SOD or against yeast proteins, other modifications have been introduced in the ELISAs and RIBAs, including the use of recombinant antigens produced in *E. coli* as fusion products with proteins other than SOD. Most of the RIBAs are not confirmatory assays in the strict sense, since they do not involve neutralisation and are really only a variation on the ELISA theme. They are more correctly described as "supplemental" assays.

Although the currently available anti-HCV tests are much more reliable, problems of interpreting positive and negative results remain. The antibodies detected by these assays are not neutralising antibodies. On its own, therefore, a positive anti-HCV test gives no indication of whether the patient has had an acute infection and has recovered completely or whether there is ongoing (chronic) infection. In contrast to HBV and HDV, it has not yet been possible to identify or detect circulating HCV antigens that might distinguish between patients with chronic infection and those who have cleared the virus and in whom the anti-HCV antibodies are merely a reflection of past infection.

Detection of circulating viral genomic material (HCV-RNA) is currently the only serological method of determining whether a positive anti-HCV test result reflects active HCV infection. This requires use of the reverse transcription polymerase chain reaction (PCR) technique, which has become the "gold standard" and is described in detail in Chapters 3 and 4. It is necessary to use such a sensitive technique because viraemia in HCV infections usually occurs at only very low levels. Nonetheless, seronegativity for HCV-RNA does not exclude

ongoing HCV infection because the viraemia fluctuates and patients can be seronegative for HCV-RNA while continuing to harbour the virus in their livers or at extrahepatic sites (Romeo et al. 1993; Myhre et al. 1994; Saleh et al. 1994b; Barrera et al. 1995; Blajchman et al. 1995; Koskinas et al. 1995; Pereira et al. 1995; Schmidt et al. 1995).

Using a combination of PCR for HCV-RNA, a second-generation anti-HCV test (ELISA 2, Ortho Diagnostic Systems) incorporating the c22, c33 and c100 peptides and a four-antigen RIBA comprising these and the 5-1-1 peptide (Chiron Corp.), Barrera et al. (1995) have recently described some of the serological events following transfusion-related HCV infection. By following 41 patients who developed post-transfusion NANB hepatitis for eight years, these investigators defined three main patterns of outcome (Fig. 1.10). At onset of the hepatitis, only 22 (54%) were seropositive for any of the anti-HCV antibodies sought, although within one month 94% had seroconverted and by six months all were seropositive. Ten patients had an uncomplicated ("self-limiting") acute hepatitis with apparent complete recovery as evidenced by sustained normalisation of serum alanine aminotransferase (ALT) activities, but liver biopsies were not performed. In these cases, the incubation period was significantly shorter and the patients had significantly higher ALT activities during the initial acute hepatitis than the other 31 patients who progressed to chronic liver disease. However, five of the ten patients with apparently self-limiting hepatitis continued to have fluctuating viraemia throughout the follow-up period despite persistently normal ALT activities and, although they eventually lost anti-c100 antibodies, they remained seropositive for anti-c22 and anti-c33. In the five who appeared to completely clear the virus, the various anti-HCV antibodies persisted for several years at declining titres before they all eventually disappeared. Whether complete disappearance by rigorous testing for antibodies against all of these HCV peptides does reflect complete recovery from infection is, however, still uncertain. Barrera et al. (1995) did not specify the limits of detection of the PCR assay for HCV-RNA that they used but Yuki et al. (1994), using a very sensitive quantitative PCR assay, have shown that about 60% of patients with low levels of viraemia do not have antibodies against the c22, c33, c100, 5-1-1 peptides or against proteins derived from the NS5 region.

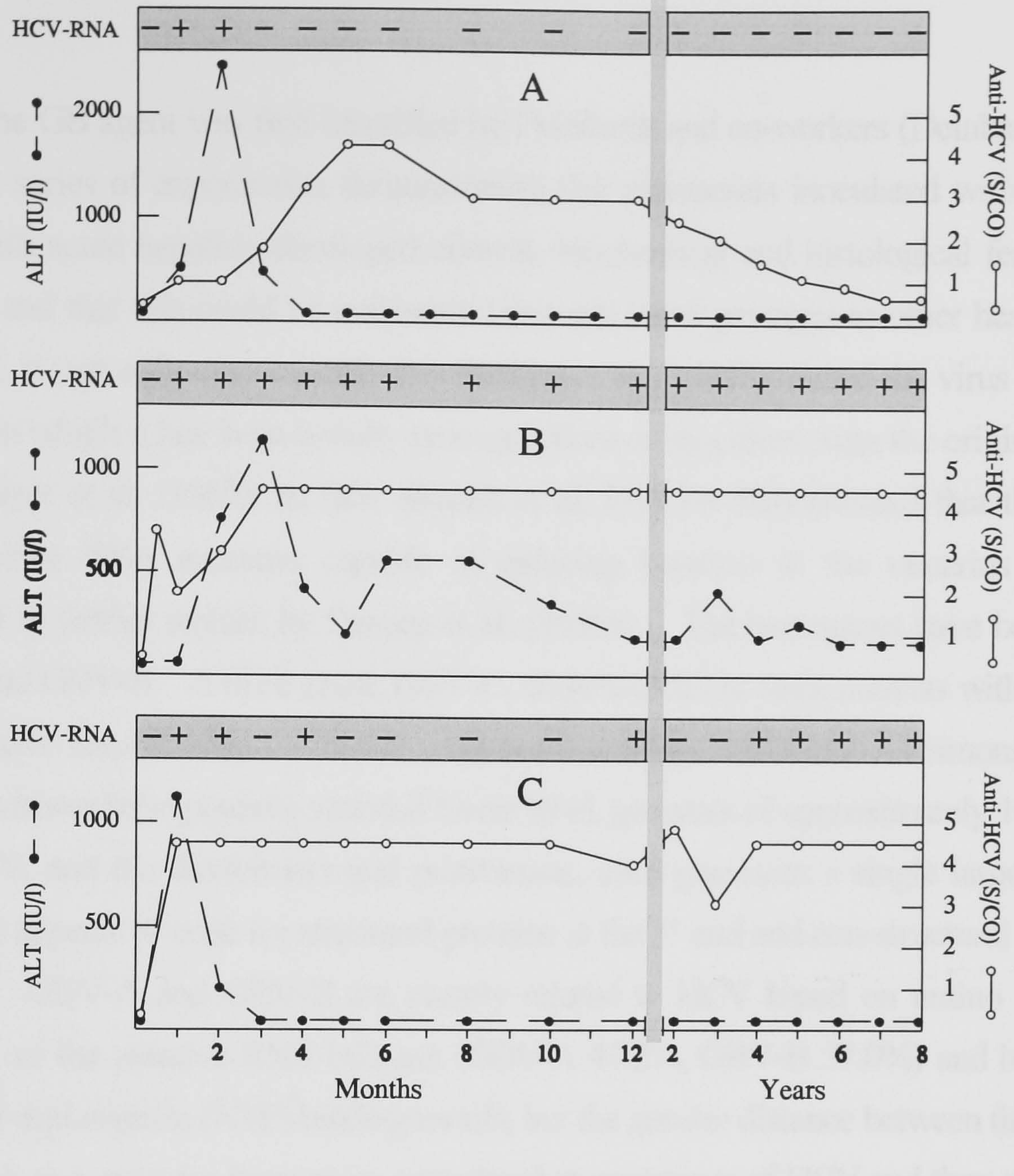


Figure 1.10 **Serological events associated with different outcomes of transfusion-related HCV infection.** Panel A = Acute infection with complete recovery and clearance of the virus. Panel B = Chronic infection with fluctuating serum alanine aminotransferase (ALT) activities. Panel C = Persistent infection with fluctuating viraemia and normal ALT activities. Anti-HCV antibody results are shown as the signal:cut-off (S/CO) ratios in the ELISAs. *Adapted from*(Barrera et al. 1995)

The serology of HCV infection is still evolving. To date, all of the commercial anti-HCV assays measure only IgG antibodies to the various peptides. However, Yuki et al. (1995) have recently shown that IgM antibodies to the core protein show a significant correlation with levels of viraemia, and Quiroga et al. (1995) have suggested that this isotype of anti-HCV may be useful in the assessment of HCV replication and in monitoring biochemical and virological responses to interferon treatment. As happened in the early years during development of the serology of HAV, HBV and HDV, therefore, it is likely that new tests will evolve as more information about the natural history of HCV and host responses to the virus become available.

1.5 THE HEPATITIS GB VIRUSES

The GB agent was first identified by Deinhardt and co-workers (Deinhardt et al. 1967) through a series of experiments demonstrating that marmosets inoculated with serum from a patient with acute hepatitis developed clinical, biochemical and histological features of acute hepatitis, and that this could be transmitted through serial passages to other healthy monkeys. However, it was only very recently that Simons et al. (1995b) cloned the virus from serum of tamarins in which it had been serially passaged from an inoculum from the original serum used by Deinhardt et al. (1967). In fact, Simons et al. (1995b) demonstrated that there were two flavivirus-like RNA genomes capable of inducing hepatitis in the tamarins and this was confirmed in further studies by Simons et al. (1995b). The two agents have been designated GBV-A and GBV-B. A third agent, GBV-C, demonstrated in some patients with hepatitis, was found to have considerable nucleotide sequence homology with GBV-A (Simons et al. 1995a). All three viruses have positive stranded linear RNA genomes of approximately 10 kb in length. Like HCV, and the flaviviruses and pestiviruses, each possesses a single large open reading frame that appears to code for structural proteins at the 5' end and non-structural proteins at the 3' end. GBV-A and GBV-B are closely related to HCV based on amino acid sequence homology of the putative RNA helicase (GBV-A 47.2%, GBV-B 57.0%) and have conserved nucleotide triphosphate (NTP) binding motifs, but the genetic distance between the three viruses and HCV is too great for them to be considered as genotypes of HCV and they show sufficient divergence from HCV at the amino acid sequence level to indicate that they form a separate and distinct viral group.

Evidence relating to the clinical significance of the GB viruses is still emerging, but they appear to be an important cause of fulminant non-A-E hepatitis (Yoshiba et al. 1995). Initial serological surveys of the prevalence of antibodies to the viruses indicate that their geographical distribution is similar to that of HCV, with nearly a third of African patients showing evidence of exposure to GBV but much lower (about 2%) rates among one study of blood donors in the USA. However, there seem to be important differences within populations (Zuckerman, 1995). Patients with HCV infection seem to be at increased risk of GBV-C infection, with the highest incidence (24%) being recorded in HCV-infected intravenous drug users (Aikawa et al. 1996).

CHAPTER 2

RATIONALE AND PLAN OF INVESTIGATIONS

2.1 RATIONALE

2.2 PLAN OF INVESTIGATION

2.3 PROGRESS OF INVESTIGATION

2.1 RATIONALE

As noted in Chapter 1(Section 1.1), the original aim of the present study was to investigate the impact of chronic viral hepatitis on hepatic schistosomiasis in Libya. When the study began in 1992, little was known about the background prevalence of viral hepatitis in that country and it was therefore considered essential to begin by collecting serum samples from subjects without evidence of Schistosomiasis to provide controls for these investigations. Sera were obtained from 301 apparently healthy volunteers and from 52 hospitalised patients(16 with a diagnosis of chronic non-A, non-B hepatitis and 36 undergoing regular haemodialysis for chronic renal failure who had received multiple blood transfusions). The serum samples were frozen immediately and shipped to the U.K. Unfortunately, this had only just been completed when the United Nations imposed sanctions against Libya. Collection and transportation of further frozen samples became impracticable because travel was possible only via a roundabout route through Malta, Egypt, and/or Tunisia. However, preliminary screening of the samples that had been collected revealed that a very high proportion (35.2%) of the "healthy" subjects had at least one marker of exposure to the hepatitis B virus (HBV), with 11.6% actively infected (HbsAg seropositive)at the time of testing, and that 6% had evidence of exposure to the hepatitis C virus (anti-HCV seropositive). Additionally, nearly 90% of the multiply-transfused haemodialysis patients had evidence of exposure to HBV and/or HCV.

In view of the above, it was decided to use the material collected to undertake a detailed study of hepatitis viral infection in this population. Since detailed follow-up to determine clinical outcome in any cases of acute infection was impossible, it was decided to concentrate the study on those viruses (B, C and D) known to be capable of chronic infection, with particular reference to hepatitis C. Although this would clearly not constitute a full epidemiological survey, it was felt that the results might provide useful information about viral hepatitis in Libya.

2.2 PLAN OF INVESTIGATION

In addition to screening for the full range of HBV markers (HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc), it was decided to test all HBsAg-positive subjects for HBV-DNA to determine how many had actively replicating virus, and for antibodies against the hepatitis delta virus (HDV) to obtain information about the frequency of concomitant HDV infection in this

population. Anti-HCV positive subjects would be re-tested for antibodies against a range of epitopes in a panel of synthetic peptides corresponding to gene products of different parts of the HCV genome, for purposes of confirming results of the initial screening assay and to determine whether any of these epitopes appeared to be immunodominant. HCV-RNA would be determined by the polymerase chain reaction(PCR) to assess how many had active HCV infection, and HCV genotypes would be determined in all HCV-RNA positive individuals to obtain information about which variants of the virus were prevalent in Libya. Results would be correlated with demographic parameters, clinical findings, serum biochemical liver tests and, where possible, with severity of disease assessed histologically.

2.3 PROGRESS OF INVESTIGATION

Once the initial screening and other investigations relating to the prevalence of hepatitis B, C and D virus infection in this Libyan population was satisfactorily completed, the study progressed to genotyping of all HCV-RNA seropositive individuals. It was decided that, partly for control purposes and partly for purposes of comparison of the geographical distribution of the various genotypes, it would be useful to also genotype approximately 100 patients with chronic hepatitis C from other parts of the world who were attending the Institute for Liver Studies. Initially, the genotyping was undertaken by restriction fragment length polymorphism (RFLP) analysis but, as this method was not capable of defining subtypes of the virus, the studies were repeated using the type-specific primer(TSP)"nested"PCR method of Okamoto et al (1992). This was not found to be particularly satisfactory, mainly because a large number of the isolates could not be assigned specific types, and the process was repeated again using type - specific primers obtained from Dr. Masashi Mizokami in Nagoya, Japan. Sequencing of the 5'UTR, core and NS5 regions of the HCV genomic material in these isolates was then undertaken and phylogenetic analysis was performed to determine whether there were any new HCV variants among the various isolates and to resolve questions about the results of genotyping by various methods used.

Finally, during the latter stages of the study, the opportunity arose to collaborate with Dr. Mizokami to investigate the prevalence of the GBV hepatitis in the Libyan subjects, and these data have been included.

CHAPTER 3

SUBJECTS & METHODS

3.1 SUBJECTS

3.2 METHODS

3.2.1 Serum Biochemical Liver Tests

3.2.2 Hepatitis B and D Viral Serology

3.2.2.1 Testing for hepatitis B (HBV) viral markers

3.2.2.2 Screening for hepatitis D

3.2.3 Hepatitis C Virus (HCV) Serology

3.2.3.1 Anti-HCV antibodies

3.2.3.2 Detection of HCV-RNA by polymerase chain reaction (PCR)

RNA extraction

Reverse transcription

Polymerase chain reaction (PCR)

3.2.3.3 Southern blotting of PCR products

3.2.4 Genotyping the hepatitis C virus

3.2.4.1 Typing by restriction fragment length polymorphism (RFLP) analysis

3.2.4.2 Typing by the method of Okamoto et al

3.2.4.3 Typing by the type-specific primer (TSP) method

3.2.5 Sequencing of hepatitis C virus cDNA

3.2.6 GBV-C virus detection

3.2.7 Phylogenetic analysis

3.2.8 Liver histology

3.2.9 Statistical analyses

3.1 SUBJECTS

A total of 353 indigenous Libyan subjects was studied. These comprised 301 apparently healthy individuals, 36 patients with chronic renal failure undergoing regular haemodialysis who had received multiple blood transfusions from Libyan donors, and 16 patients with non-alcoholic liver disease who had never received blood transfusions and in whom the working diagnosis was chronic non-A, non-B viral hepatitis. Haemophiliacs were excluded because they had received blood products from abroad. Full details of these subjects are given in Chapter 5.

Serum from 10 ml of blood from each subject was immediately separated, frozen in 1 ml aliquots and stored at -70°C. The samples were then transported to the U.K. in dry ice and stored at -20°C until used for the various investigations detailed in Chapters 5 to 8. All subjects gave informed consent to clinical examination at the time of sampling, with particular attention to signs and symptoms of liver disease (jaundice, ascites, hepatomegaly, splenomegaly, and cutaneous stigmata).

For purposes of comparison with Libyan patients with hepatitis C virus (HCV) infection, an additional 106 consecutive patients from other parts of the world with chronic hepatitis C, who were attending the Institute of Liver Studies, were also studied. Details of these patients are given in Chapter 6.

3.2 METHODS

3.2.1 Serum Biochemical Liver Tests

Total serum bilirubin, alkaline phosphatase, gammaglutamyl transferase, total protein and albumin were determined by standard automated techniques (SMAC, Technitron) by the routine chemical pathology services of the Libyan hospitals involved. Full blood count and prothrombin time (by the Quik technique [Quik, 1935]), were performed by the routine haematology departments of these hospitals.

Serum aspartate (AST) and alanine (ALT) aminotransferase activities were determined

on the stored sera at the Institute of Liver Studies by colorimetric assays (505[AST/GOT] and 505-P[ALT/GPT], respectively, from Sigma Chemical Co., Poole, Dorset). The measurements were made strictly according to the manufacturer's instructions and using the standard curve provided, with an upper normal limit of 50 IU/l for both assays.

3.2.2 Hepatitis B and D Viral Serology

3.2.2.1 Testing for hepatitis B (HBV) viral markers

Screening for infection with hepatitis B virus (HBV) was performed initially by the Red Crescent Blood Transfusion service in Libya by testing for HBV surface antigen (HBsAg) using the Wellcotest haemagglutination assay (Wellcome Diagnostics, Beckenham, Kent, UK). All samples were re-tested in the Institute of Liver Studies for HBsAg and 'e' antigen (HBeAg), and for antibodies against HBeAg (anti-HBe), HBsAg (anti-HBs) and the HBV core antigen (anti-HBc) by commercial automated enzyme immunoassays ("IMx", Abbott Laboratories, Chicago, Ill., USA) with the kind assistance of Ms. Heather Smith, Principal Biochemist.

Hepatitis B viral DNA (HBV-DNA) was detected by using a commercial manual molecular hybridization assay (Abbott Laboratories, Chicago, Ill., USA) according to the manufacturer's instructions. Briefly, the procedure involves treating 100 µl of serum with proteinase to release any protein-encapsulated DNA in the sample. Any DNA present is then uncoiled by addition of sodium hydroxide in sodium chloride solution and incubating the sample overnight at 65°C with a ^{125}I-labelled probe comprising a short nucleotide sequence corresponding to the HBsAg-encoding region of the HBV genome, which will hybridize with any HBV-DNA present. The sample is then passed over a small (1.5 cm diameter) column of sepharose and washed through with a Tris buffer, to separate unbound probe (which is retained by the column) from the probe/HBV-DNA complex. The latter passes through the column with the first column volume of wash buffer and is collected and radioactivity in the sample determined by counting (in the present studies using a Packard Cobra II autogamma counter). Specimens with counts equal to or greater than the manufacturer's cut-off value are considered positive for HBV-DNA, which is then quantified and expressed in pg HBV-DNA/ml by calculation with a formula derived from a standard curve provided by the manufacturer.

3.2.2.2 Screening for hepatitis D

Total (IgM, IgG and IgA) antibodies against the delta virus antigen (HDAg) were sought by a commercial enzyme immunoassay (Sorin/Biomedica, Italy). This is a competitive ELISA in which anti-HDAg antibodies in the patient's serum compete with a peroxidase-conjugated human anti-HDAg antibody for binding to HDAg. Briefly, purified HDAg is added to the wells of microtitre plates coated with a monoclonal anti-HDAg antibody which "captures" the HDAg. Excess HDAg is washed off and 10 µl of patient's serum is added, followed immediately by 100 µl of the peroxidase-labelled anti-HDAg. The plates are incubated overnight at room temperature, then the wells are aspirated and washed. To detect binding of the peroxidase anti-HDAg to the immobilized HDAg, 100 µl of tetramethyl benzidine in 0.005% hydrogen peroxide is added. After incubation for 30 min at room temperature, the enzyme reaction is stopped by addition of 100 µl of the manufacturer's blocking reagent (sulphuric acid). The intensity of the resulting color reaction is quantified by spectrophotometric measurement of the absorbance at 450 nm. Anti-HDAg antibodies in the patient's serum competing with the peroxidase-anti-HDAg for binding to the HDAg on the plate cause a reduction in the intensity of the colour developed. Positive results are defined in relation to the absorbance values given by the manufacturer's positive and negative controls, which are run concurrently.

3.2.3 Hepatitis C Virus (HCV) Serology

3.2.3.1 Anti-HCV antibodies

For the reasons discussed in detail in Chapter 4, screening of sera for anti-HCV antibodies in the present study was performed with a commercial second/third generation enzyme immunoassay (HCV-EIA, United Biomedical Inc., Raritan, NJ, USA). This assay employs two synthetic peptides immobilized on microtitre plates - one corresponding to a segment of the highly conserved core region of the HCV genome (see Chapter 1, Fig. 1.6) and the other to an overlapping part of the NS3/NS4 region(Hosein et al. 1991). Samples giving positive reactions in this assay (defined by reference to the manufacturer's positive and negative controls run concurrently) were re-tested in duplicate to confirm the positive result. In addition, each anti-HCV positive sample was further tested in five separate ELISAs (produced by United Biomedical Inc.) for antibodies against these two peptides individually and against additional synthetic peptides corresponding to the envelope (E1/E2), NS1 and NS5 regions of the genome.

All assays were performed exactly according to the manufacturer's instructions using the positive and negative controls supplied. Briefly, sera are incubated in the antigen-coated microtitre plates at 37°C for between 15 min and 1 hour (depending on which assay is being used) and the plates are then washed. Antibodies binding to the antigens in the wells are then detected by adding peroxidase-conjugated anti-human IgG, incubating for 15 min to 1 hour at 37°C, washing thoroughly, then adding either O-phenylenediamine-HCl or tetramethyl benzidine as instructed and incubating for the same period as above. Reactions are stopped by addition of 1N sulphuric acid and the absorbance of the colour produced is read at 492 or 450 nm, as appropriate.

3.2.3.2 Detection of HCV-RNA by polymerase chain reaction (PCR)

RNA extraction

Total RNA from serum was extracted by the guanidinium thiocyanate/phenol/ chloroform method of (Chomczynski and Sacchi, 1987) using RNAzol B (Biogenesis, Bournemouth, UK) according to the manufacturer's instructions. Briefly, 200 µl of RNAzol was added to 100 µl of serum in a 1.5 ml polypropylene microcentrifuge (Eppendorf) tube and the mixture was incubated with continuous vortex mixing for 15 min at 4°C. Chloroform (30 µl) was then added to disrupt any viral envelope present and, after mixing for 1 min, the sample was centrifuged at 13,000 rpm for 15 min to separate the aqueous and non-aqueous phases. The RNA-containing aqueous phase was then carefully pipetted into a fresh, sterile, Eppendorf tube and the RNA was precipitated by adding an equal volume of isopropanol containing 20 µg of glycogen as a carrier, incubating for 1 hour at -20°C, then centrifuging at 13,000 rpm for 15 min. The resulting RNA pellet was washed twice with cold 70% ethanol and dried under vacuum. Two normal human sera and two sera from patients known to have chronic hepatitis C were similarly processed as negative and positive controls, respectively, concurrently throughout. As additional internal "assay controls", parallel experiments were run using the primers DM151 and DM152 (Table 3.2) supplied with the GeneAmp kit produced by Perkin-Elmer/Cetus. The rationale for use of these conditions, and those of the procedures described below, is discussed in Chapter 4 (Section 4.2.2).

Reverse transcription

DNA complementary (cDNA) to the freshly extracted RNA was synthesised by reverse transcription using Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT, Promega,

Southampton, UK). RNA pellets from above were resuspended in 20 µl sterile water treated with diethylpyrocarbonate (DEPC) and heated at 65°C for 10 min to destroy secondary structure. 2.0 µl of the RNA suspension was then added to 8 µl of a mixture containing 1 mmole/l of each of the four deoxynucleotide triphosphates (adjusted with NaOH to pH 7.0), 5 mM $MgCl_2$, and 10-fold strength (10X) RT buffer (500 mM KCl, 100 mM Tris-HCL, pH 8.3). The mixture was overlaid with 2 drops of mineral oil (Sigma) and incubated at 70°C for 10 min to reduce RNA secondary structure formation. To the cooled sample was then added 100 units of MMLV-RT, 20 units of recombinant RNAase inhibitor (Promega), random hexamers (hexameric nucleotides containing all possible combinations of G, A, T and C), and DEPC-treated water to give a final volume of 20 µl. This "reverse transcriptase mixture" (Table 3.1) was incubated for 30 min at 42°C, then for 5 min at 98°C to inactivate the MMLV-RT. After the cDNA synthesis, all samples were boiled for 30 min to ensure complete denaturation of the reverse transcriptase - any residual activity of which could give rise to false-positive results in the subsequent PCR steps. To avoid contamination, the recommendations of (Kwok and Higuchi, 1989) were strictly followed. In addition, preparation of samples, performance of PCR and subsequent analysis of the products were each conducted in separate laboratories.

Table 3.1 Composition of the "reverse transcriptase mixture".

Component	Volume	Final concentration
$MgCl_2$	4.0 µl	5 mM
10X RT buffer	2.0 µl	1X
DEPC-water	5.5 µl	-
dATP	1.0 µl	2 mM
dCTP	1.0 µl	2 mM
dGTP	1.0 µl	2 mM
dTTP	1.0 µl	2 mM
RNase inhibitor	1.0 µl	100 U/ml
Random hexamers	1.0 µl	2.5 µM
MMLV-RT	0.5 µl	125 U/ml
RNA extract	2.0 µl	-
Total volume	20 µl	-

Polymerase chain reaction (PCR)

To amplify the cDNA produced above "nested" PCR (the principles of which are described in Chapter 4) was performed according to (Saiki et al. 1988) using primers corresponding to segments of the highly conserved 5' untranslated region (5'UTR) of the HCV genome (Okamoto et al. 1990). The procedure was optimised by starting the reactions "hot", with a preliminary incubation of the sample at 85°C prior to addition of *Taq* polymerase (Perkin-Elmer/Cetus, Norwalk, CT, USA), as suggested by (Erlich et al. 1991). The first round of PCR was performed with the "outer" primers JR12 and JR19 described by (Ulrich et al. 1991): the downstream (3') primer designated JR19 representing nucleotides 197-216 of the HCV genome, and the upstream (5') primer JR12 representing nucleotides 1-20 (Table 3.2). The "inner" primers JR13 and JR14(Romeo et al. 1993) corresponding respectively to nucleotides 35-53 and 14-161 were used in the second round. All of the primers and the oligonucleotide probe used for Southern blotting (below) were synthesised and sequences validated by the Department of Molecular Medicine, King's College School of Medicine & Dentistry, London.

Table 3.2 "Nested" primer pairs used in the polymerase chain reaction

Primers	Position *	Sequence (5'-3')
"Outer":		
JR12	nt 1-20	GGCGACACTCCACCATAGAT
JR19	nt 197-216	CGCCCAAATCTCCAGGCATT
"Inner":		
JR13	nt 35-53	GAACTACTGTCTTCACGCA
JR14	nt 140-161	GGCAATTCCGGTGTACTCACC
Controls:		
DM151	-	GTCTCTGAATCAGAAATCCTTCTATC
DM152	-	CATGTCAAATTTCACTGCTTCATCC

* Corresponding to nucleotides (nt) in 5'UTR of HCV genome

For the first round of PCR, to 5.0 µl of the cDNA sample in a sterile 1.5 ml Eppendorf tube was added 5.0 µl of 10-fold strength (10X) PCR buffer (200 mM Tris-HCl, pH 8.8, 500 mM KCl, 15 mM $MgCl_2$, 0.5% Triton X-100), 1.0 µl of an equimolar mixture of the four deoxyribonucleotides (dNTPs: dATP, dCTP, dGTP, dTTP), 0.25 µl each of the outer sense and

antisense primers, *Taq* DNA polymerase and DEPC-water to give a total volume of 50 µl (Table 3.3). The sample was then immediately subjected to 25 cycles of denaturation at 94°C for 35 sec, primer annealing at 50°C for 40 sec, and primer extension at 68°C for 150 sec in an automatic thermal cycler (Perkin-Elmer/Cetus). The amplified DNA was then stored at 4°C overnight or at -20°C if longer storage was anticipated.

Table 3.3 Composition of polymerase chain reaction mixtures

Component	First round PCR		Second round PCR	
	Volume	Final concentration	Volume	Final concentration
Initial cDNA sample	5.0 µl	-	-	-
First round product	-	-	1.0 µl	-
10X PCR buffer	5.0 µl	1X	2.0 µl	1X
dNTP mixture	1.0 µl	2 mM	0.2 µl	1 mM
Outer primers	0.5 µl	4-16 pmol/ml	-	-
Inner primers	-	-	0.2 µl	1.5-6.0 pmol/ml
Taq DNA polymerase	0.25 µl	20 U/ml	0.2 µl	20 U/ml
DEPC-water	38.25 µl	-	16.4 µl	-
Total volume	50 µl	-	20 µl	-

For the second round of amplification, 1.0 µl of the first round product in a total 20 µl of reaction mix (Table 3.3) was subjected to a further 25 cycles as above. The entire sample was then electrophoresed in a 3% Nusieve gel (Gibco), equivalent to 1% agarose, at 100V for 1 hour in TAE buffer (40 mM Tris, 10 mM sodium acetate, 1 mM EDTA, 28 mM acetic acid), stained with ethidium bromide and examined under UV light. Molecular size markers (123 ladder, Gibco) spanning the range of expected bands of the PCR product were run on each gel. All PCR assays were performed in duplicate with positive, negative and internal "assay" controls for all steps as noted above. Interpretation of results is discussed in Chapter 4 (Section 4.2.2).

3.2.3.3 Southern blotting of PCR products

The specificity of the HCV-RNA detection by PCR was confirmed in all cases by Southern blotting using a ^{32}P-labelled synthetic oligonucleotide probe with a sequence (nt 116-136 WRL 757 5'-GAGGCCATAGTGGTCTGCG-3') internal to, and not inclusive of, that of the inner nested primers. The oligonucleotide bands obtained by PCR were transferred electrophoretically to nylon membranes (Hybond N+, Amersham, Bucks., UK) using a dot blot apparatus (Bio-Rad, Hemel Hempstead, Herts., UK) according to the manufacturer's instructions. The membranes were pre-hybridised by incubation at 65°C for 4 hours in 5-fold strength (5X) Denhardt's solution (5g Ficoll, 5g polyvinylpyrrolidine, 5g bovine serum albumin in 100 ml), with 5X SSPE (20X SSPE = 3 M NaCl, 0.2 M $NaH_2PO_4.H_2O$, 20 mM EDTA) 0.5% sodium dodecyl sulphate (SDS) and 200 µg/ml sonicated salmon sperm DNA before addition of the radiolabelled probe and overnight hybridisation at 40°C. The membranes were then successively washed at room temperature with 5X SSPE for 10 min, 2X SSPE with 0.1% SDS for 10 min, 1X SSPE with 0.1% SDS for 10 min, and finally with 0.2X SSPE with 0.1% SDS at 65°C for 10 min. After drying at 80°C, the membranes were autoradiographed at room temperature using Hyperfilm ßmax (Amersham) for 48 hours.

3.2.4 Genotyping the hepatitis C virus

3.2.4.1 Typing by restriction fragment length polymorphism (RFLP) analysis

RNA was extracted from 100 µl of serum and cDNA prepared by reverse transcription as described above. The cDNA was then amplified by PCR as above but using the outer primers 209 and 939 shown in Table 3.4 for the first round of amplification and the inner primers 211 and 940 for the second round(Garson et al. 1990b). This was done in the presence of 10% dimethylsulphoxide (DMSO) for 30 cycles of the following program: 1 min at 94°C, 1.5 min at 45°C, 3 min at 68°C. The resulting PCR products were electrophoresed in 3% low melting point agarose gels and the oligonucleotide fragments were detected by ethidium bromide staining and ultraviolet illumination as above.

RFLP analysis was performed by digesting 25 µl of the second round PCR product overnight at 37°C. The reaction was carried out in the presence of with 10 units in 5 µl 10-fold strength enzyme reaction buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) of each of the restriction endonucleases *HaeIII/RsaI*, *HinfI/MunI* or *MvaII* in a total volume of 30 µl of 10X enzyme reaction buffer. The digestion products were visualized under UV light after electrophoresis in 4% Metaphor agarose gel (FMC BioProducts, Sittingbourne, Kent, UK) in Tris-borate buffer (134 mM Tris-HCl, 68 mM sodium borate, 25 mM disodium ethylene diamine tetraacetate (EDTA), pH 10.0) containing 0.5 µg/ml ethidium bromide. The oligonucleotide fragments were sized by comparison with the migration distances of standard DNA markers (50-1000 bp size range; AMS Biotechnology (UK) Ltd., Witney, Oxon) and results were interpreted as described in Chapters 4 and 6.

Table 3.4 Details of primers used for genotyping of the 5'UTR region of the HCV genome by RFLP analysis.

Primer No.	Position * (nt)	Polarity	Sequence (5'-3')
209	8 to -21	-	ATACTCGAGGTGCACGGTCTACGAGACCT
939	-297 to -278	+	CTGTGAGGAACTACTGTCTT
940	- 279 to -260	+	TTCACGCAGAAAGCGTCTAG
211	-54 to -29	-	CACTCTCGAGCACCCTATCAGGCAGT

* Numbering from start of main open reading frame.

3.2.4.2 Typing by the method of Okamoto et al

The strategy for genotyping of HCV by the "nested" PCR method of Okamoto et al. (1992b) is discussed in Chapter 4 (Section 4.4). In the present study, the procedure described by these investigators was followed precisely. cDNA from the RNA extracted from 100 µl of serum was reverse transcribed, and the first round of PCR was performed for 35 cycles as above, but using the universal primers Nos. 186 and 256 (Table 3.5). 1.0 µl of the product was then subjected to a second round of PCR for 30 cycles of: 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min. In this second round, the universal primer No. 104 was used together with a mixture of four type-specific antisense primers: Nos. 132 to 135, Table 3.5. The products were

then subjected to electrophoresis in 2% agarose gels made from 1.5% Nusieve and 1.5% SeaKem (Gibco, Paisley, UK), equivalent to 2% agarose, stained with ethidium bromide and examined under UV light.

Table 3.5 Details of primers used for genotyping of the HCV core region by the method of Okamoto et al.

Primer No.	Position * (nt)	Polarity	Sequence (5'-3')
104	148-167	+	AGGAAGACTTCCGAGCGGTC
186	391-410	+	ATGTACCCCATGAGGTCGGC
256	139-158	+	CGCGCGACTAGGAAGACTTC
132	185-204	-	TGCCTTGGGGATAGGCTGAC
133	272-291	-	GAGCCATCCTGCCCACCCCA
134	302-321	-	CCAAGAGGGACGGGAACCTC
135	251-270	-	ACCCTCGTTTCCGTACAGAG

* Numbering from start of main open reading frame.

3.2.4.3 Typing and sub-typing by the type-specific primer (TSP) method

The strategy used for typing and sub-typing by the TSP method, and interpretation of results, are discussed in Chapters 4 (Section 4.4) and 7, respectively. RNA was extracted from 100 µl of serum, reverse-transcribed and subjected to two rounds of PCR as above. The primers used for each round are detailed in Table 3.6. In the first round, 5.0 µl of the resulting cDNA was amplified through 20 cycles of: 94°C for 1 min, 45°C for 1 min and 72°C for 1 min, using the outer primers Sc2 and Ac2. In the second round, two aliquots of 1.0 µl of the first round product were amplified for a further 20 cycles of: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, using a mixture of primers S7, S2a, G1b, G2a, G2b and G3b (Mix 1 - for genotypes 1b, 2a, 2B and 3b) with one aliquot and primers S7, G1a, G3a, G4a, G5a and G6a (Mix 2 - for genotypes 1a, 3a, 4, 5 and 6) with the other.

Table 3.6 Details of primers used for genotyping of HCV by the TSP method.

Primer *	Sequence (5'-3') †	Position ‡
FIRST ROUND PCR		
Sc2	GGGAGGTCTCGTAGACCGTGCACCATG	-24 to +3
Ac2	GAG(AC)GG(GT)AT(AG)TACCCCATGAG(AG)TCGGC	417 to 391
SECOND ROUND PCR - MIX 1		
S7	AGACCGTGCACCATGAGCAC	-12 to +8
S2a	AACACTAACCGTCGCCCACAA	40 to 60
G1b	CCTGCCCTCGGGTTGGCTA(AG)	222 to 203
G2a	CACGTGGCTGGGATCGCTCC	178 to 159
G2b	GGCCCCAATTAGGACGAGAC	325 to 306
G3b	CGCTCGGAAGTCTTACGTAC	164 to 145
SECOND ROUND PCR - MIX 2		
S7	AGACCGTGCACCATGAGCAC	-12 to +8
G1a	GGATAGGCTGACGTCTACCT	196 to 177
G3a	GCCCAGGACCGGCCTTCGCT	220 to 201
G4a	CCCGGGAACTTAACGTCCAT	77 to 58
G5a	GAACCTCGGGGGGAGAGCAA	308 to 289
G6a	GGTCATTGGGGCCCCAATGT	334 to 315

* Notations 1a to 6a are according to the nomeclature of Simmonds et al.(1994a).
† Parentheses indicate degenerate nucleotide pairs
‡ Numbering is from the start of the main open reading frame

3.2.5 Sequencing of hepatitis C virus genomic material

Viral sequences were amplified by reverse transcription and PCR from 100 µl of serum as above (Section 3.2.3.2), except that one of the inner primers (No. 211, Table 3.4) was biotinylated (supplied by the Dept. of Molecular Medicine as above). 40 µl of a suspension of streptavidin-coated magnetic beads ("Dynabeads", Dynal Ltd., Wirral, Merseyside, UK) which had been previously washed with 40 µl of 0.1% aqueous bovine serum albumin were placed in a 1.5 ml Eppendorf tube. 40 µl of the second round PCR product was added to the beads and incubated for 20 min at room temperature with occasional stirring. The beads were then pelleted

with a magnet, washed with 40 µl of extraction buffer (10 mM Tris/HCl, pH 7.5, with 1 mM EDTA and 2 M NaCl), resuspended in 8 µl of 0.15M NaOH and incubated for 10 min at room temperature to separate the double-stranded DNA. The beads, with attached single-stranded antisense DNA were again pelleted by magnet, and the supernatant (containing unbound single-stranded sense DNA) was removed and stored. Finally, the beads were washed once with 50 µl of NaOH, once with 50 µl of extraction buffer, and once with 50 µl of TE buffer (10 mM Tris/HCl with 1 mM EDTA, pH 7.5) to remove traces of the supernatant, then resuspended in 20 µl of TE buffer.

Sequencing of the single-stranded PCR products was performed using the "Sequenase" kit supplied by United States Biochemicals (Cleveland, Ohio, USA) according to the manufacturer's instructions but with slight modifications to reduce secondary structure formation, namely, by carrying out the reactions in 10% DMSO and heat-denaturing the DNA template before primer annealing(Winship, 1989). For sequencing of the HCV core region, primers 954 and 410 (Table 3.6) were used in the first round and 953 and 410 in the second round. For sequencing of the 5'UTR region, primers 940 and 211 (Table 3.4) were used.

For each sequencing reaction, 1 µl DMSO, 2 µl of reaction buffer, 10 ng of each of the appropriate primers in 2 µl, and 5 µl of either the final Dynabead suspension or the supernatant from the above PCR were mixed together in a 1.5 ml Eppendorf tube and incubated for 3 min at 60°C. After allowing the tube to cool to room temperature, it was transferred to an ice/water bath and DMSO was added to a final concentration of 10%. 2.5 µl of each of the four dideoxynucleotide (ddn) termination mix solutions (80 µM dATP, dCTP, dGTP and dTTP, supplemented with 8 µM ddnTP in 50 mM NaCl) were pipetted into separate wells of a 96-well round bottomed microtitre plate and warmed to 37°C in a water bath. To the DNA sample in the Eppendorf tube was added 1 µl of 0.1% dithiothreitol (DTT), 1 µl of labelling mix (dATP, dCTP, dGTP and dTTP, 600 µM each), 1 µl α-[^{35}S]-dATP (approximately 20 µCi, Amersham), and 2 units of T7 DNA polymerase (Promega) in 1 µl of TE buffer. After mixing, 3 µl of the DNA sample was then added to each of the four termination mixtures in the microtitre plate and incubation continued for 5 min. Reactions were terminated by addition of 4 µl of 95% formamide containing 20 mM EDTA, 0.05% Xylene and 0.05% bromophenol blue.

Table 3.7 **Details of primers used for sequencing of the core and NS5 regions of the HCV genome.**

Primer	HCV genomic region	Position (nt) *	Polarity	Sequence (5'-3')
410	Core	410 to 391	-	ATGTACCCCATGAGGTCGGC
954	Core	330 to 349	+	ACGCCGGGGGT(AG)CATGGCCCA
953	Core	-21 to +3	+	AGGTCTCGTAGACCGTGCACCATG
955	Core	118 to 140	-	GCAGGGGCCCCAGGTTGGGTGTG
242	NS5	8304 to 8275	-	GGCGGAATTCCTGGTCATAGCCTCCGTGAA
555	NS5	8227 to 8208	-	CCACGACTAGATCATCTCCG
243	NS5	7904 to 7934	+	TGGGGATCCCGTATGATACCCGCTGCTTTGA
554	NS5	7935 to 7958	+	CTCAACCGTCACTGAACAGGACAT

* Numbering from start of main open reading frame.

The reaction products from the above were heated for 5 min at 90°C, allowed to cool, then electrophoresed in an 8% denaturing polyacrylamide gel containing 5% urea and 0.02% N,N,N',N'-tetramethylethylenediamine (TEMED), in TBE buffer (890 mM Tris-Borate, 890 mM Boric acid, 20 mM EDTA) at 40-60 watts. The gel was then fixed with two changes of 1 litre of 5% acetic acid with 5% methanol, dried on a gel drying apparatus and autoradiographed for 3 days as above (Section 3.2.3.3). To determine the genotype from sequence data, individual sequences were compared to the EMBL database using the BLAST alignment search program (Altschul and Lipman, 1990; Karlin and Altschul, 1990). Interpretation of results is discussed in Chapter 8.

3.2.6 Hepatitis G virus detection

Detection of hepatitis GBV-C genomic material in serum samples in the present study was kindly performed by Dr. Masashi Mizokami of Nagoya City University Medical School, Japan, using cDNA produced by reverse transcription with the primers shown in Table 3.8 and a hemi-nested PCR protocol very similar to that described above. Briefly, 1 µl of the cDNA was amplified with 20 pmol each of the antisense primer MUGR1 and the sense primer G8 (Table 3.8) in a mixture containing 200 µM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl,

1.5 mM $MgCl_2$ and 0.001% gelatin, for 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. A hemi-nested second round of PCR amplification was done by using 1 µl of the first round product with the sense primer G8 and antisense primer MUGR2 (Table 3.8). Ten µl of the second round product was then electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed. The expected size of the hemi-nested PCR products to be derived from GBV-C genomic RNA by this procedure is 140 bp. Two positive and two negative controls were included in each assay run. For confirmation, the amplification products were inserted into M13 phage vectors and three independent clones of each product were sequenced.

Table 3.8 Details of primers used for detection of hepatitis GB virus

Primer	Polarity	Sequence
G8	+	5'-TATGGGCATGGAATACCCCT-3'
MUGR1	-	5'-TCTTTGATGATAGAACTGTC-3'
MUGR2	-	5-'TCCTTACCCCTA(G)TAATAGGC-3'

3.2.7 Phylogenetic analysis

The nucleotide sequences determined from the sequencing procedure described above were formatted and analysed initially by programs available in the University of Wisconsin Genetics Computer Group (GCG) sequence analysis package, version 8.0 (Devereux et al. 1984). All sequences were edited using the sequences editor, aligned by the PILEUP program and then formatted using the multiple sequence formatting (MSF) file in the GCG package. Distances between pairs of sequences were estimated using the DNADIST program of the PHYLIP version 3.4 package kindly provided by Dr. J. Felsenstein (Felsenstein, 1991), using a model which allows for different rates of transition and transversion and different frequencies of the four nucleotides. Phylogenetic trees were constructed using the neighbour-joining algorithm with the NEIGHBOR program. Equivalent phylogenetic relationships were determined by maximum likelihood analysis with the DNAML program and 2000 bootstrap replicates of neighbour-joining trees with the SEQBOOT and CONSENSE programs, in the PHYLIP package. The rationale for phylogenetic analysis is discussed in Chapter 4.

3.2.8 Liver histology

Histological examination of liver biopsies in the present study was kindly undertaken by Dr. Bernard Portmann, Consultant Histopathologist in the Institute of Liver Studies, King's College Hospital, London, who reported on the degree of necroinflammatory activity and whether there was evidence of fibrosis or established cirrhosis. Necroinflammatory activity was semiquantitatively classified as follows:

Inactive - inflammatory infiltrate, if present, mild and confined to portal tracts.

Mild - moderate inflammatory inflitrate in portal tracts, with occasional foci in lobules, but with no hepatocellular necrosis.

Moderate - dense inflammatory infiltrate in the majority of the portal tracts, with disruption of limiting plates in some and periportal piecemeal necrosis, but without significant lobular activity or bridging necrosis.

Severe - dense inflammatory infiltrate in all portal tracts, with disruption of the limiting plates in the majority and periportal piecemeal necrosis extending into lobules with bridging necrosis.

However, because it is difficult to precisely define disease activity on the basis of a single liver biopsy taken at one time point and because, in some instances, classification under these four categories would have led to small numbers of patients in some groups, in the present study patients were broadly classified as having either inactive/mild or moderate/severe disease for purposes of analysis.

3.2.9 Statistical Analyses

The methods for statistical treatment of data for phylogenetic analysis were incorporated within the computer program packages as described above (Section 3.2.7) and in Chapter 8. Statistical analyses of other data were performed by using non-parametric tests: the Mann-Whitney U test for continuous variables, and the Chi-Square test with Yates' correction for small number for dichotomous variables. Differences were considered statistically significant when $p < 0.05$ by two-tailed analysis.

CHAPTER 4

SELECTION, MODIFICATION AND VALIDATION OF METHODS

4.1 DIAGNOSIS OF HEPATITIS B AND D VIRUS INFECTION

4.1.1 - Validation of commercial HBV-DNA Assay

4.2 DIAGNOSIS OF HEPATITIS C VIRUS (HCV) INFECTIONS

4.2.1 Enzyme-linked immunoassays (ELISAs) for anti-HCV antibodies
4.2.2 Polymerase chain reaction (PCR)

4.3 DIAGNOSIS OF HEPATITIS GB INFECTION

4.4 GENOTYPING

4.5 SEQUENCING AND PHYLOGENETIC ANALYSIS

4.1 DIAGNOSIS OF HEPATITIS B AND D VIRUS INFECTION

Hepatitis B (HBV) and Delta (HDV) virus infections are usually associated with the presence in serum of one or more of a wide range of viral proteins, antibodies and other specific markers of these viruses, and the rationale for selection of appropriate parameters for investigation of HBV and HDV infections is related to the serological events that normally follow exposure to these viruses - discussed in detail in Chapter 1 (Section 1.3.4).

As noted in Chapters 3 (Section 3.2.2.1) and 5 (Section 5.1.2), for economic reasons testing for HBV infection in Libya is undertaken using only the Wellcotest haemagglutination assay for HBsAg. This was one of the earliest tests to become commercially available and is generally regarded as relatively insensitive. Furthermore, since part of the purpose of the present study was to gather background information on the extent of previous exposure to the virus in this population, it was decided to screen all sera for the full range of markers available, using the automated Abbott IMx system (Section 3.2.2.1). To obtain additional information about the status of infection in HBsAg-positive subjects, HBV-DNA in serum samples from these individuals was sought using the Abbott molecular hybridization assay. At the time, this was a relatively new technique that had not been widely used and it was felt important to validate it. This was done by comparing it with an "in house" spot hybridization assay (see below). The commercial HBV-DNA and its validation were kindly performed for the present study by Dr. Leila Pereira of the Institute of Liver Studies, King's College Hospital, London.

Since Delta virus infections almost invariably occur against a background of HBV infection (Section 1.3.2), and suppression of HBV very rarely extends to complete disappearance of HBsAg, it was decided to screen for HDV only in subjects who were found to be HBsAg seropositive and to test initially only for *total* anti-HDAg antibodies (Section 3.2.2.2). Any patients found to be positive for these antibodies would then be further investigated by testing for IgM anti-HDag to determine whether they had current HDV infection.

4.1.1 - Validation of Commercial HBV-DNA Assay

The spot hybridization assay for HBV-DNA described by (Fagan et al. 1985)was used, with some modifications, for validation of the commercial HBV-DNA assay. Double-stranded DNA was partially purified from 50 µl of serum from an HBV carrier by layering it on 600 µl of 30% sucrose (in 0.01 M Tris, 0.05 M NaCl, 0.002 M EDTA, 0.1% bovine serum albumin (BSA) and 0.1% mercaptoethanol) in 2 ml polycarbonate tubes, incubating overnight at 4°C and centrifuging at 25,000 rpm for 4.25 hours at 4°C. The resulting pellets, containing the DNA, were resuspended in 50 µl of a 10-fold strength (10X) sodium citrate buffer (SSC: single strength (1X) = 0.15 M NaCl, 0.015 M Na-citrate) and incubated for 15 min at 37°C. The DNA was then denatured by mixing 50 µl of the suspension with 100 µl of 1 M NaOH and incubating at room temperature for 10 min. 200 µl of this mixture was applied to a pre-determined spot on a pencil-drawn grid on a Gene Screen Hybridization filter (Gene Screen™, Du Pont, USA), which had been pre-soaked in 2X SSC for 10 min at room temperature. The DNA was fixed to the filter by drying under gentle vacuum (~20 mmHg) on a 96-well manifold (Bethesda Research Labs, Cambridge, UK) and washed with 200 µl of 0.5 M Tris-HCl in 3 M NaCl (pH 7.4), containing two drops of 0.6% bromophenol blue (in 20% glycerol, 0.5% SDS, 0.2 M Tris, 0.005 M EDTA, 0.1 M Na-acetate, pH 8.2) for subsequent identification of the points of application of samples on the screen. The filter was then dried, first for 1 hour at room temperature and finally at 80°C for two hours.

The dried membrane filters were pre-hybridized by incubating overnight in glass cylinders in a rotary oven at 42°C with 200 µl per cm^2 of membrane of 6X SSC containing 50% formamide, 0.5% SDS, 10 µg/ml denatured salmon sperm DNA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA. Hybridization was effected using 100 µl of this solution/cm^2 of membrane containing denatured ^{32}P-labelled HBV-DNA and incubating at 42°C overnight in the rotary oven. The membranes were washed twice for 5 min in 2X SSC at room temperature, twice for 30 min at 65°C in 2X SSC with 1% SDS, and finally for 30 min in 0.1X SSC at room temperature, then dried at room temperature and autoradiographed for 48 hours at -70°C using Kodak X-Omat film to identify positive spots. To quantify the HBV-DNA in the samples, the filter grid squares were cut out, placed in counting vials containing 4 ml water and ^{32}P radioactivity determined by Cerenkov radiation counting (Scintran, BDH, Poole, Dorset, UK) in a TRI-CARB 460C/460CD counter (Packard Corp., USA). Results were

expressed in picograms of HBV-DNA by reference to a standard curve prepared using known amounts of cloned HBV-DNA run in parallel.

Sera from six patients (who were not part of the present study) that had been found to be positive by the commercial HBV-DNA assay were selected to represent a range of HBV-DNA concentrations and, together with six randomly selected sera that were negative by this assay, were re-tested using the spot hybridization assay. The negative sera were all found to be negative also by the latter assay. Results from the two assays for the positive sera showed a good correlation (Fig. 4.1), although the slope of the regression line suggests that the spot hybridization assay may be more reliable at higher HBV-DNA concentrations.

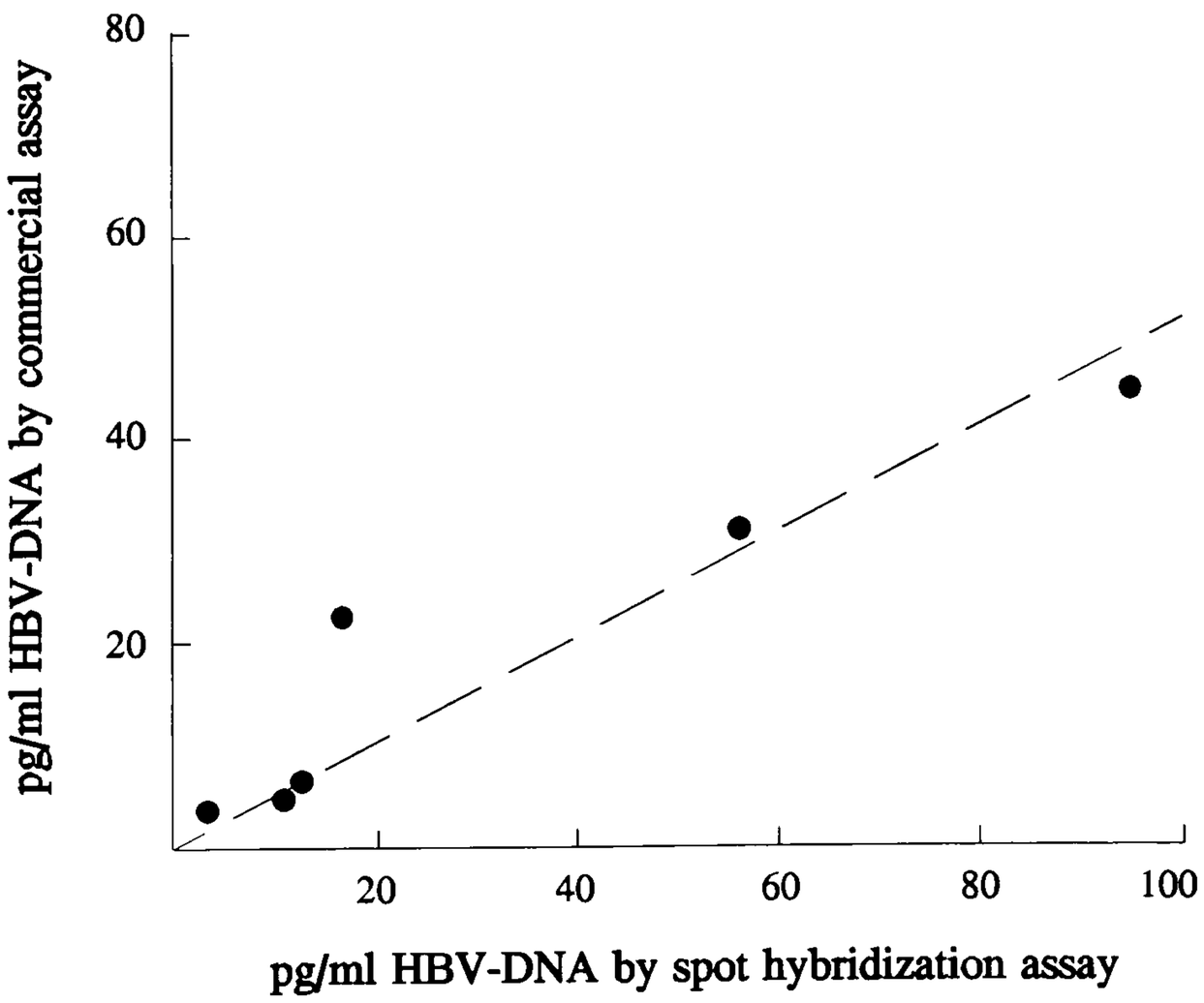


Figure 4.1 **Comparison between the spot hybridization and commercial HBV-DNA assays**

4.2 DIAGNOSIS OF HEPATITIS C VIRUS (HCV) INFECTIONS

4.2.1 Enzyme-linked Immunoassays (ELISAs) for Anti-HCV Antibodies

As noted in Chapter 1 (Section 1.4.4), the reliability of the early tests for anti-HCV antibodies had been called into question. At the time that the present study was begun in 1992 improved, second/third generation, anti-HCV antibody assays were beginning to become available but were quite costly. United Biomedical Inc. (UBI: Raritan, NJ, USA) had developed a series of enzyme immunoassays (ELISAs) based on synthetic peptides (Hosein et al. 1991): a screening assay using peptides corresponding to products of the core (c22) and NS3/NS4 regions of the HCV genome (Chapter 1, Fig. 1.7), and a series of supplemental assays employing the individual peptides derived from these and from the envelope, NS1 and NS5 regions. The manufacturer very kindly offered to provide the assay kits for this research at no cost.

Apart from the considerable cost saving, the main advantage of employing the UBI system was that, by using synthetic peptides, it avoided problems of false-positivity due to antibodies in patients' sera that react with the fusion proteins used for the recombinant peptides (Ikeda et al. 1990) or with residual microbial proteins (from the microbial expression systems) that might have escaped purification of the recombinant antigens. An added advantage was that, perhaps because it employed synthetic peptides (or for other reasons not divulged by the manufacturer), it seemed to be a generally "cleaner" assay in that it had a very low cut-off to define positivity. With almost all ELISA plate readers, the linear range of optical density (OD) readings reaches a maximum at between 2.0 and 2.5 OD units. The UBI assay had a maximum cut-off of about 0.15 OD units and, consequently, positive/negative (P/N) ratios of 12-20 were usually observed with strongly positive sera. In contrast, the other assays available at the time (the Ortho, Innotest, and Abbott systems) had cut-offs of 0.4 - 0.6 OD units, which gave maximum P/N ratios of only 4 - 6. Thus, the UBI system allowed for much greater discrimination between positivity and negativity, particularly with less strongly reactive samples.

For the above reasons, it was decided to use the UBI system throughout the present studies employing the screening assay for initial testing of sera and the ELISAs against the individual peptides as "confirmatory" tests. By way of validation, the UBI screening assay was compared with a second-generation Abbott HCV-EIA, based on recombinant c22 and c200 peptides (Chapter 1, Fig. 1.7). Sera from eight HCV-RNA seropositive patients with chronic hepatitis C and 10 sera from autoimmune hepatitis patients with hypergammaglobulinaemia who were seronegative for HCV-RNA were tested simulataneously by the two assays. From Fig. 4.2 it can be seen that there was good agreement between the assays although, as noted above, clearer discrimination was obtained with the UBI assay.

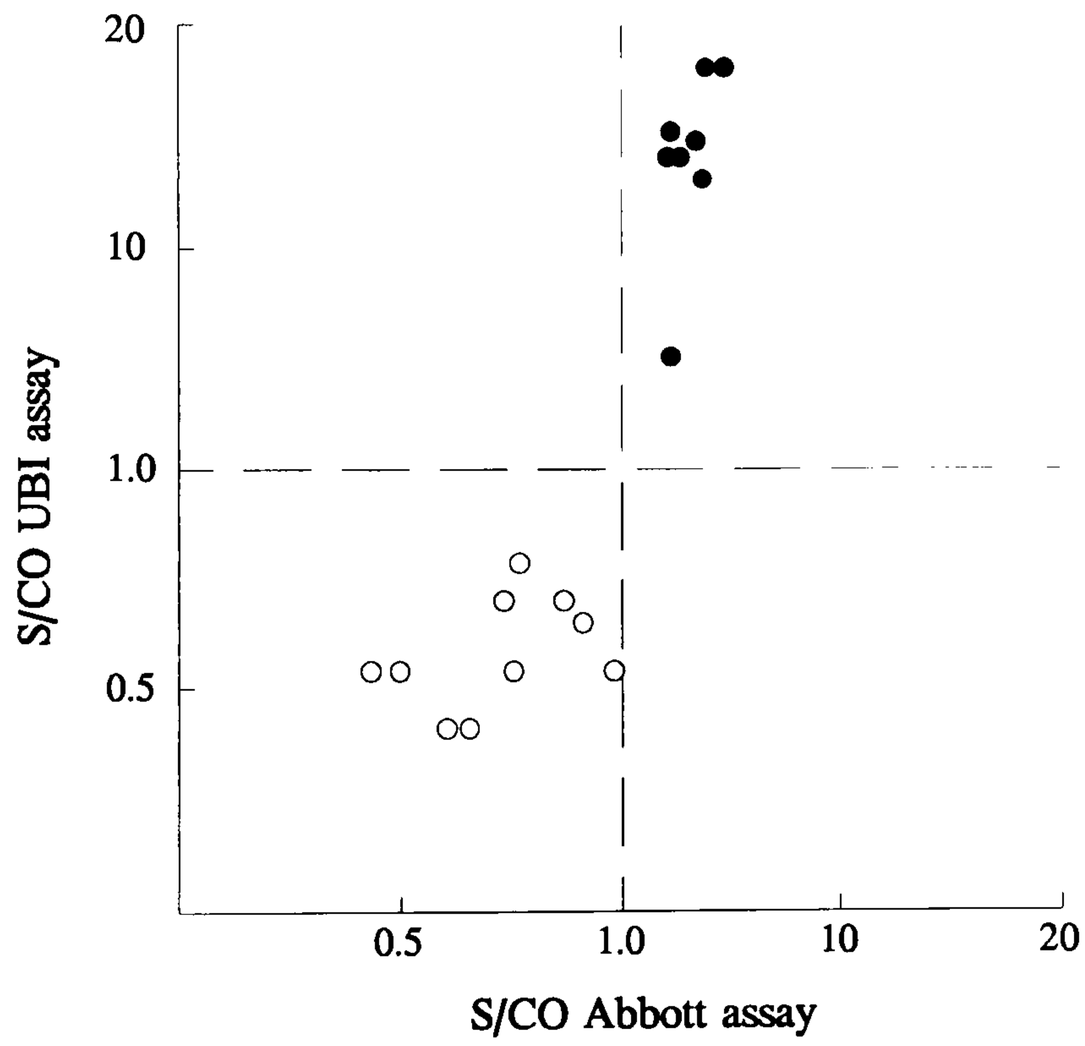


Figure 4.2 **Comparison between the United Biomedical Inc. (UBI) and Abbott anti-HCV antibody ELISAs.** Results are shown as sample/cut-off (S/CO) ratios calculated from the observed optical densities (ODs) in relation to the manufacturers' recommended cut-offs for positivity (broken lines). HCV-RNA seropositive sera from patients with chronic hepatitis C are indicated by closed circles, while open circles show results for HCV-RNA negative sera from patients with autoimmune hepatitis and hypergammaglobulinaemia.

4.2.2 Polymerase Chain Reaction (PCR)

The development of the PCR process is one of the most important technical advances in molecular biology in the past decade (Scharf et al. 1986; Mullis and Faloona, 1987; Erlich, 1989; Saiki et al. 1988). It is the only method whereby the very small amounts of circulating hepatitis C virus genomic material (HCV-RNA) can be detected which, as discussed in Chapter-1 (Section 1.4.4), is necessary in order to distinguish between on-going and previous infections in patients with anti-HCV antibodies.

Amplification by PCR enables production of sufficient material for separation by gel electrophoresis and visualization of a characteristic band under ultraviolet light after staining with ethidium bromide (Chapter 3, Section 3.2.3.2). For confirmation of a positive result, the electrophoresed product may be transferred to a nitrocellulose sheet (Southern blotting) and the band identified by probing with a radiolabelled or chemiluminescent nucleotide sequence corresponding to part of the HCV genome (see Section 3.2.3.3). Further confirmation may be obtained by sequencing the amplified product to ensure that its nucleotide sequence corresponds to that of HCV-RNA. The PCR process itself and these confirmatory procedures are exceedingly laborious. Sequencing of the amplified product from large numbers of sera is therefore almost never undertaken on a routine basis and even Southern blotting is usually performed as a control only on a proportion of samples under test. However, in the present study it was decided to confirm all PCR-positive results by Southern blotting and sequencing was also undertaken because of the need to obtain information about genotypes of the virus.

The first step in the PCR procedure, as applied to the detection of HCV-RNA in serum, involves extraction of the total RNA in the specimen. Complementary DNA (cDNA) copies of all extracted RNA are then made, using an exogenous reverse transcriptase. To detect cDNA copies that are specifically complementary to HCV-RNA, the cDNA product is amplified through 30 to 60 cycles by use of a DNA polymerase and the appropriate mixture of individual nucleotides, together with a "primer" comprising a short nucleotide sequence corresponding to part of the HCV genome. In the present study, to increase specificity, the so-called "nested" PCR technique (Garson et al. 1990a; Romeo et al. 1993) was used, whereby amplification is performed in two stages with two separate pairs of primers: an "inner" and an

"outer" set, the nucleotide sequences of which correspond to parts of the highly conserved 5'-untranslated (5'UTR) segment of the HCV genome.

Several laboratories around the world have been evaluating the ideal reagents and conditions for the development of a sensitive and specific PCR assay for HCV-RNA, and there are several minor (and a few major) variations between the different centres. In the present study, the procedure adopted represents an amalgam of conditions and reagents reported by the different centres to give optimal results. Firstly, for the initial extraction of RNA from serum samples, it was decided to use the commercially available guanidinium thiocyanate/phenol/ chloroform reagent RNAzol™ B produced by Biogenesis because it is a high quality reagent designed for simple RNA isolation. On mixing with an aqueous solution such as serum, it forms a two-phase system. RNA complexes with the guanidinium and water and remains in the aqueous phase. Hydrophilic interactions of DNA and proteins are abolished and these components partition with the organic phase. Inclusion of a blue dye in the reagent facilitates aqueous/organic phase identification. The system allows for the isolation of all RNA species, including small RNAs, in a pure undegraded form protected from ribonucleases. An added advantage is that it is a one-step procedure that takes only about 1.5 hours to perform.

The PCR technique used here allows for the detection of as few as 100 viral RNA molecules per ml of serum (see (Ulrich et al. 1993), and below). However, as with all PCR processes, this extreme sensitivity is also a major disadvantage because the risk of false positive results due to contamination of the samples is very high, and great care has to be taken to avoid this (Kwok and Higuchi, 1989). False positive reactions from carry-over of previously amplified DNA in reagents or on equipment, and from cross-contamination with true positive samples, are the main complications(Kwok and Higuchi, 1989). Contamination can also arise from foreign unamplified DNA (from sloughed skin cells or aerosols) entering the reaction vessel. In addition to the usual rules of common sense generally applied to molecular biological techniques, therefore, several specific precautions were taken in the present study to avoid these problems. Thus, the RNA extraction and amplification steps were performed in separate rooms, reagents were prepared in a PCR product-free environment, autoclaved, and stored in aliquots that were used only once, and cDNA samples were added last to each tube. Additionally, dedicated equipment and consumables were set aside for all PCR work, with separate sets of automatic positive displacement pipettes and sterile pipette tips for each

procedure, and great care was taken not to allow tubes to become contaminated during insertion of the reagents. To detect cross-contamination, samples containing PCR mix without templates during the amplification process (negative controls) and two positive controls were run concurrently in each batch of assays. In addition, the positive controls were regularly checked by colleagues in the laboratory and every sample was tested at least twice to confirm the validity of the results. All PCRs were also carried out with the positive "assay controls" provided by Perkin-Elmer/Cetus, as a check that the assays were working properly.

Several other precautions were taken to avoid erroneous results. To prevent direct interference with *Taq* polymerase by the reverse transcriptase after the cDNA synthesis from the extracted RNA, all cDNA products were heated at 98°C for 10 min to inactivate any residual reverse transcriptase prior to amplification. Amplification requires multiple, carefully controlled cycles of heating, to separate (melt) the double-stranded DNA to allow for copying of the two strands by the DNA polymerase, and cooling (to anneal the newly-synthesised strands). The temperatures range from ambient to nearly 100°C during the cycles and markedly influence the rates of reactions in the system. Thus, accurate temperature control is essential. To achieve this, the fully automatic Perkin-Elmer/Cetus DNA Thermal Cycler was used in the present studies. Reactions were carried out in 0.5 ml polypropylene tubes placed in the wells of the thermal cycler heating block. The rate of heat transfer can be influenced by variations in thickness of the walls of tubes and of the air spaces between the tubes and the block. In the present study, tubes from a single supplier were used throughout (to ensure uniformity of wall thickness) and were always carefully pressed down into the heating block to reduce the air space between the tube and the block in order to enhance efficient and reproducible heat transfer.

Conventional three-temperature PCR protocols require heating the cDNA sample at 92-97°C to denature double-stranded DNA into two single strands. Because of the high number of G/C bonds in the cDNA, melting at 97°C for the first five cycles is normally required to produce the single stranded template for the PCR reaction but, in subsequent cycles, a melting temperature of 94°C is used because the smaller PCR product usually melts completely at a lower temperature than the starting cDNA. The temperature is then reduced to 50°C to permit annealing of the target-specific primers onto the single-stranded DNA. Finally, the temperature is raised again to 68°C (the optimum reaction temperature for *Taq*

polymerase) to add complementary nucleotides and thereby extend the primers to produce two copies of the original target strands. In the present study, the initial five cycles at 97°C was replaced by the "hot start" procedure involving a preliminary incubation at 85°C for 10 min which, as suggested by (Erlich et al. 1991), is optimal for reducing formation of secondary structures and for denaturing any residual reverse transcriptase. The PCR was then repeated 25 times in the first round with the outer primers (Chapter 3, Section 3.2.3.2). Then, 1 µl of the resulting reaction product mixture was transferred to a second tube containing the inner primers and subjected to a further 25 cycles for the second round of amplification. In our laboratories, it was found that the addition of more than 1 µl to the second round PCR reaction leads to the appearance of multiple non-specific bands in the product, which can reduce the sensitivity of the PCR.

The selection of the genomic sequences to be used in designing the primers for detection of HCV-RNA by PCR is also crucial. Such sequences must be well conserved in different HCV variants to ensure good sensitivity and, to avoid non-specificity, should not be present in other viruses. The non-translated segment comprising approximately 324 nucleotides at the 5' end of the long open reading frame of the HCV genome has been found to be the region that best fulfils these requirements(Garson et al. 1990a; Okamoto et al. 1990). A number of different primer sequences based on this region have been used in different laboratories but the ones chosen for the present study (detailed in Chapter 3, Table 3.2) are reportedly the most sensitive and specific (Romeo et al. 1993; Ulrich et al. 1990; Garson et al. 1991).

As noted above, the PCR procedure used in the present studies is extremely sensitive. Theoretically, the number of copies of the target sequence doubles with each cycle of the reaction. This could produce up to 10^{19} - 10^{20} copies of any HCV-RNA present in the specimen under test but, in practice, the yield with this protocol is only approximately 10^{12} copies(Ivinson, 1991; Sallie, 1993). There are several reasons for this apparent shortfall. Firstly, it is only after the third cycle that there is a doubling of the defined sequence. Secondly, The DNA polymerase is only relatively heat stable (Gelfand and White, 1990) and repeated exposure to the high denaturating temperature progressively inactivates the enzyme, resulting in plateauing of the growth in copy numbers. Also, with successive cycles, there is a progressive decrease in the concentrations of the nucleotide substrates required for the new

strand synthesis and these become rate-limiting. Nevertheless, an amplification factor of 10^{12} copies is more than adequate for most purposes.

With protocols where incubation times at each temperature are very short, e.g. < 30 seconds, the sample temperature may not reach the optimum required for denaturation and annealing, and lower yields will be obtained. The timing of the synthesis step is one of the most critical. Too short a time will not allow complete synthesis of the new strand, whereas too long a time will encourage non-specific amplification. The incubation times used in the present study (35 sec for denaturation, 40 sec for annealing and 150 sec for extension) were determined by a process of trial and error to be the optimum times required for maximum yield of specific product under the conditions employed and with the primers used.

Finally, during the course of this study, a number of techniques for quantitative measurement of HCV-RNA levels became available. These include the quantitative chemiluminescent PCR recently described by (Whitby and Garson, 1995) and the branched DNA (bDNA) assay, which represents a new approach to detection of nucleic acid directly in clinical samples at physiological concentrations (Urdea et al. 1991; Alter et al. 1995). The latter has been applied to the detection of cytomegalovirus, HBV and HIV, and has been used by several investigators for monitoring viral load during antiviral treatment in chronic HCV infection but, while it gives good reproducibility, it seems to be less sensitive than standard PCR for detection of HCV in that it reportedly detects only 72% of PCR-positive samples(Lau et al. 1993). However, as it was felt that quantitation of HCV-RNA would add little to the present findings, it was decided not to repeat the studies using these techniques.

4.3 DIAGNOSIS OF HEPATITIS GB INFECTION

Tests for anti-GBV antibodies are not yet widely available. Therefore, in the present study, it was necessary to rely only on PCR to detect GBV genomic material in the sera of the subjects studied. Because of the close similarity between GBV-A and GBV-C (Chapter 1, Section 1.5), it was decided to use primers corresponding to sections of the 5'UTR region of GBV-C according to Yoshiba et al. (1995)as described in Chapter 3 (Section 3.2.6).

4.4 GENOTYPING

Traditionally, viruses have been classified according to serotypes. Attempts are being made to do this with HCV (Stuyver et al. 1993; Simmonds et al. 1993c) and serological methods for HCV typing, based on the ability to distinguish antibodies elicited by infection with different genotypes (without using PCR-based methods), have been developed and have been used to detect antibodies against group-specific recombinant proteins in the putative NS4 (Bhattacherjee et al. 1995; Kao et al. 1996) and NS5(Simmonds et al. 1993a) protein regions. However, there are not yet sufficient data to make global comparisons. Accordingly, the present study has relied only on genotyping for identifying HCV types in Libya and comparing these with types in other parts of the world.

Methods for genotyping HCV are still evolving. Those that have been most widely used so far include restriction fragment length polymorphism (RFLP) analysis, type-specific probing (TSP), a reverse hybridization assay (line probe assay, LIPA) based on the highly conserved 5'UTR, and nucleotide sequencing. Each has a number of advantages and disadvantages. The LIPA allows for typing and subtyping of the most common genotypes (Stuyver et al. 1993), but it has been suggested that about 8% of cases would be mistyped by this method (Maertens et al. 1993). The development of typing assays based on RFLP requires considerable amounts of comparative sequence data, because there is some intratypic sequence variability that may result in each genotype showing more than one electropherotype. For this reason, it is necessary to compare the electropherotypes obtained with different combinations of restriction endonucleases, but previous methods have not always proved reliable. For example, by comparing the electropherotypes produced after digestion with the restriction

enzyme combinations *Hae*III-*Rsa*I and *Scr*FI it is possible to identify HCV genotypes 1, 2 and 3 (McOmish et al. 1993) but, at the outset of the present study, it was found that type 4 showed the same electrophoretic pattern as type 1 in this system. This was overcome by using *Mva III-Hinf I* instead of *ScrFI* , as described by Davidson et al. (1995).

The TSP method, first described by Okamoto et al (1992) using primers corresponding to sections of the core region (Chapter 3, Table 3.5), was devised for identifying genotypes 1a, 1b, 2a and 2b. Because RFLP does not allow for identification of sub-types of the virus, it was decided to apply Okamoto's method in the present study. In the first round of PCR a fragment of 276 bp is generated with a mixture of primers based on highly conserved sequences and the second round , with mixtures of subtype-specific primers generates subtype-specific amplification products(Okamoto et al. 1992b). The best results are obtained when each HCV sub-type is separately amplified. However, a high number (sometimes up to 20%) of apparent 1a and 1b co-infections is very often found when using the nested sub-type primer 132 (Chapter 3, Table 3.5). This primer is theoretically predicted to hybridize to many 1b sequences. Normally, two mismatches lead to the difference between both sub-types at the primer 132 position. Due to non-specific priming of primer 132, it is possible to introduce a greater degree of apparent co-infections and also a greater number of unidentified types. As discussed in Chapter 7 (Section 7.3), these problems were encountered in the present study and, indeed, it was found that a large number of the HCV-positive individuals could not be sub-typed by this method. The method was therefore modified as described in Chapter 3 (Section 3.2.4.3), using primers kindly provided by Dr. Masashi Mizokami of Nagoya City University medical School, Japan.

4.5 Sequencing and Phylogenetic analysis of HCV genotypes and GBV-C

The "gold standard" for genotyping is nucleotide sequence analysis. Although not usually considered practicable for large-scale typing of clinical samples, because it is so time consuming and expensive in reagents, it was decided to undertake this for the present study because of the detailed information required and to provide the possibility of investigating whether new genotypes exist in Libya.

Nucleotide sequence analysis has played a fundamental part in virological research. However, sequencing of the entire genome of a virus requires relatively large amounts of template DNA which are often available only by virus culture *in vitro* and cloning, and the dependence on virus culture and the time required to construct and screen libraries is a major impediment to this approach. The polymerase chain reaction (PCR), which allows for amplification of DNA specifically from any viral nucleotide sequence in clinical samples, is a useful alternative that eliminates the need for prior virus isolation and cloning. Its main limitation is its dependence on detailed sequence information to specify the primers needed for amplification. Consequently, most PCR studies have to rely on comparing large numbers of sequences over a relatively short region of the viral genome and construction of libraries and screening with cloned viral DNA is likely to remain the method of choice for large sequencing projects for the foreseeable future.

Nucleotide sequencing of PCR products is carried out using the dideoxy (dd) termination method. The amplified product can be sequenced directly or it can first be cloned into a suitable plasmid or phage vector and sequenced from primers close to the cloning site. Sequencing of cloned DNA is not different from sequencing other types of recombinant DNA. Detailed protocols are described in Chapter 3 (Section 3.2.5). This method has been used successfully on a wide variety of combinations of viral DNA and primers. Double stranded DNA (either the PCR product or its cloned derivative) is heat denatured in the presence of a single primer and strand synthesis is allowed to take place initially in the presence of unlabelled dGTP, dCTP and dTTP with a limiting concentration of radiolabelled dATP, followed by incubation in the presence of each ddNTP to allow termination. A primer that has a 3' base after annealing which is one base upstream from the variable site is annealed, and a single biotinylated or radiolabelled dNTP complementary to one or other of the sequence variants is added in the presence of DNA polymerase. Addition of a labelled nucleotide to the primer takes place if the target sequence matches, whereas little or no incorporation of label occurs if a different base is present. The primer can then be separated from unreacted dNTPs, and the partial transcripts formed in each of the four termination reactions (i.e. with ddGTP, ddATP, ddTTP, and ddCTP) sized, by electrophoresis on a high-resolution denaturing polyacrylamide gel to allow the nucleotide sequence to be read. Double-stranded sequencing reactions are readable from 10-20 bp downstream from the 3' base of the sequencing primers to about 250 bp.

The nucleotide sequence data obtained by this method in the present study were subjected to phylogenetic analysis to determine the evolutionary relationships between the HCV

variants by using the various computer programs described in Chapter 3 (Section 3.2.7). At the outset, it was recognized that such analyses necessarily make a number of assumptions about the evolutionary process and that it is therefore advisable to seek concordance from a number of different methods in order to increase confidence in the results. Phylogenetic trees were inferred by using two different programs available in the PHYLIP package of Felsenstein (1993), DNAML and NEIGHBOR. The DNAML program finds the tree of the highest likelihood (maximum likelihood tree) given a particular stochastic model of molecular evolution that has been shown to perform well in simulation studies (Saitou and Nei, 1987). The global (G) option was used in the present studies because this searches a greater proportion of all possible trees. The NEIGHBOR program clusters a matrix of nucleotide distances previously estimated using the DNADIST program (Section 3.2.7). The latter was set, using the D option, to use the stochastic model that underlies DNAML in order to estimate distances corrected for the probability of multiple substitutions.

Nonetheless, the topology obtained by any of these procedures can be considered only an estimate of the phylogenetic relationships between groups of variants, and it is clearly important to know whether two groups on a tree are really distinct or whether their apparent separation falls within the error associated with estimating the tree. Most methods for estimating tree topologies do not give any information on the statistical significance of their structures. As in the present study, this is usually overcome by the use of a non-parametric statistical procedure known as "bootstrapping", which involves re-sampling the data on which the tree was based in order to generate a distribution of datasets from each of which a new tree can be determined. The frequency with which particular branches are observed in the re-sampled datasets then allows for probability statements to be attached to them in the original tree. The maximum likelihood approach has a number of advantages: it is based on an explicit model of sequence evolution; a probability statement can be associated with each interrnodal segment, as mentioned above; and the complete nucleotide sequence information is used in the determination of the phylogeny. In all cases in the present study, the maximum likelihood and neighbour joining procedures produced congruent trees.

CHAPTER 5

INVESTIGATION OF HEPATITIS B, C, D AND GB VIRUS INFECTION IN LIBYAN SUBJECTS

5.1 CONTROL SUBJECTS

5.1.1 Demographic, Clinical and Biochemical Characteristics

The 301 control subjects studied comprised 143 males and 158 females (median age 42 years, range 10-78) consecutively recruited from centres in one of three cities in Libya: 156 from Benghazi city, 115 from Tripoli and 30 from Sebha (Fig. 5.1). Seventy-six were unpaid volunteer donors attending the Red Crescent Blood Banks in these cities, who were not members of the medical or paramedical professions. They had no risk factors for viral hepatitis, i.e. no history of blood transfusions, drug misuse or contact with hepatitis, had not previously undergone surgery, did not admit to homosexuality. The remaining 225 comprised 82 medical students, 69 hospital workers, and 74 subjects attending outpatients clinics (ear/nose/throat, dermatology, obstetrics and gynaecology, superficial trauma) for minor conditions unrelated to liver disease, at the 7th October, Hawari, or Al-Jamahiya Hospitals in Benghazi city, or the Sebha General Hospital. These 225 also had none of the above risk factors for viral hepatitis.

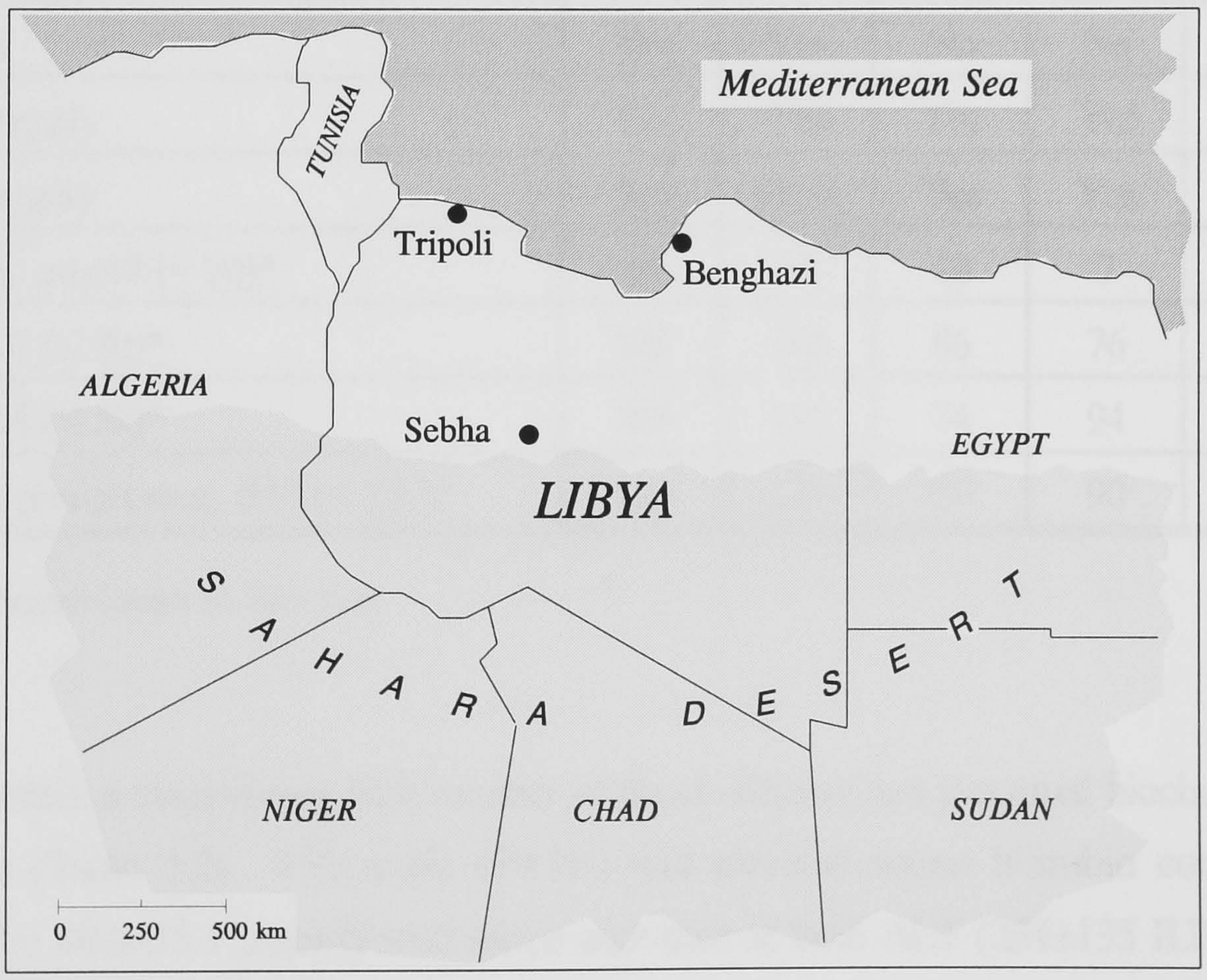


Figure 5.1 - Map of Libya showing the three locations from where subjects were recruited.

Each subject agreed to clinical examination immediately after giving blood. Although all professed to be healthy, six were found to have signs of underlying liver disease (Table 5.1). Two were clinically jaundiced with hepatosplenomegaly and elevated serum bilirubin concentrations and alanine (ALT) and aspartate (AST) aminotransferase activities. Two others had enlarged spleens on palpation (>16 cm below costal margin) with hepatomegaly and mildly elevated (≤10% above the upper normal limit) ALT and AST activities, and a further two (with normal ALT and AST activities) had splenomegaly without hepatomegaly. Other signs of chronic liver disease (ascites, spider angiomata, or palmar erythema) were not found in these six nor in any other subject.

Table 5.1 Details of the six control subjects with clinical signs of liver disease

	Subject number					
	1	2	3	4	5	6
Age	36	45	52	51	48	45
Sex	M	M	F	M	F	M
Jaundice	Yes	Yes	No	No	No	No
Hepatomegaly	Yes	Yes	Yes	Yes	No	No
Splenomegaly	Yes	Yes	Yes	Yes	Yes	Yes
Bilirubin, μmol/l (< 20)*	44	36	10	7	6	7
AST, IU/l (≤ 50)*	328	102	86	76	46	38
ALT, IU/l (≤ 50)*	254	132	74	94	42	28
Alkaline phosphatase, IU/l (≤ 120)*	155	125	101	90	65	75

* Upper normal limits in brackets

Overall, a surprisingly high number of these subjects had abnormal biochemical liver test results (Table 5.2). Fifty-eight (19.3%) had elevated serum bilirubin concentrations (mean±SD = 40.6±15.1 μmol/l) with raised activities of both ALT (254±135 IU/l) and AST (362±121 IU/l), 10 of whom also had elevated serum alkaline phosphatase activities (239±75 IU/l). A further four subjects had elevated ALT (range 80-120 IU/l)) and AST (80-160 IU/l) activities with normal serum bilirubin and alkaline phosphatase levels - making a total of 62 (20.6%) who had abnormal serum biochemical liver tests. In 22 subjects

these abnormalities were mild (≤ 10% above the upper normal limits) but in the remaining 40 they were of sufficient magnitude (55-482 IU/l, median 128) to suggest significant underlying liver disease. Interestingly, abnormal liver test results occurred more frequently in housewives (27/120 = 22.5%) than in females (5/38 = 13.2%) with other occupations, but this difference was not statistically significant (p > 0.3). However, subjects with AST and/or ALT activities > 10% above the upper normal limits were significantly older (p < 0.02) than those with abnormal values of ≤ 10% above upper normal limits (Table 5.2).

Table 5.2 **Demographic information on the 62 control subjects with abnormal biochemical liver test results.**

	No.	Number with elevated ALT or AST				Percent with abnormal ALT/AST
		≤ 10% above UNL*		> 10% above UNL*		
		ALT	AST	ALT	AST	
Sex:						
Male	143	10	10	20	27	25.9%
Female	158	12	12	20	13	20.3%
Occupation:						
Housewives	120	10	11	16	16	22.5%
Students	82	8	9	5	5	17.1%
Office workers	35	2	2	4	4	17.1%
Manual workers	34	3	3	3	3	17.6%
Others	30	1	1	2	3	13.3%
Elevated serum:						
Bilirubin	58	40	38	18	20	100%
Alk. Phos.	10	3	2	7	8	100%
Age: median years (range)		26 (16-36)		48 (30-72)		

* UNL = upper normal limit (50 IU/l)

5.1.2 Results of Hepatitis B Screening

Although all of the blood donors had been found to be seronegative for HBsAg by haemagglutination (Wellcotest: Wellcome Diagnostics, Beckenham, Kent) when tested at the Blood Banks in Libya, at the outset of the present study it was considered important to re-test all sera for HBsAg to confirm this and to test for additional HBV markers to obtain information on the extent of previous exposure to HBV. Screening of the 301 control subjects by automated ELISA (Chapter 3) revealed that 106 (35.2%) had at least one marker of hepatitis B Virus (HBV) infection (Table 5.3). Seven had anti-HBc as the only marker of exposure to HBV, one had only anti-HBs and 62 were seropostive for both anti-HBc and anti-HBs. The remaining 35 (11.6%) were seropositive for HBsAg and anti-HBc (Table 5.3). None of the HBsAg-positive subjects had circulating antibodies against the hepatitis delta virus (HDV).

Table 5.3 Frequency of hepatitis B viral (HBV) markers in the different groups of control subjects.

	No.	HBsAg positive	HBsAg negative		Total positive for any HBV marker
			Anti-HBc positive	Anti-HBs positive	
Blood donors	76	7	16	12	23 (30.3%)
Hospital workers:					
Office	35	3	7	8	11 (31.4%)
Manual	34	4	7	7	12 (35.3%)
Medical students	82	10	20	17	30 (36.6%)
Outpatients	74	11	20	19	31 (41.9%)
Totals	301	35 (11.6%)	70 (23.6%)	63 (20.9%)	106 (35.2%)

There was no significant difference in frequency of seropositivity for HBsAg or other HBV markers between the different groups of control subjects. From these data, it appears that about 35% of the general Libyan population may have been exposed to HBV.

Eleven of the 35 HBsAg-positive subjects were positive for 'e' antigen (HBeAg) and negative for anti-HBe. One of these had ongoing active viral replication as indicated by a serum HBV-DNA of 205 pg/ml (Fig.5.2). A further seven of these 35 had both HBeAg and

anti-HBe (three being also positive for HBV-DNA at 175 - 210 pg/ml), and six were anti-HBe positive without HBeAg. The remaining 11 HBsAg-positive subjects were seronegative for HBeAg, anti-HBe and HBV-DNA.

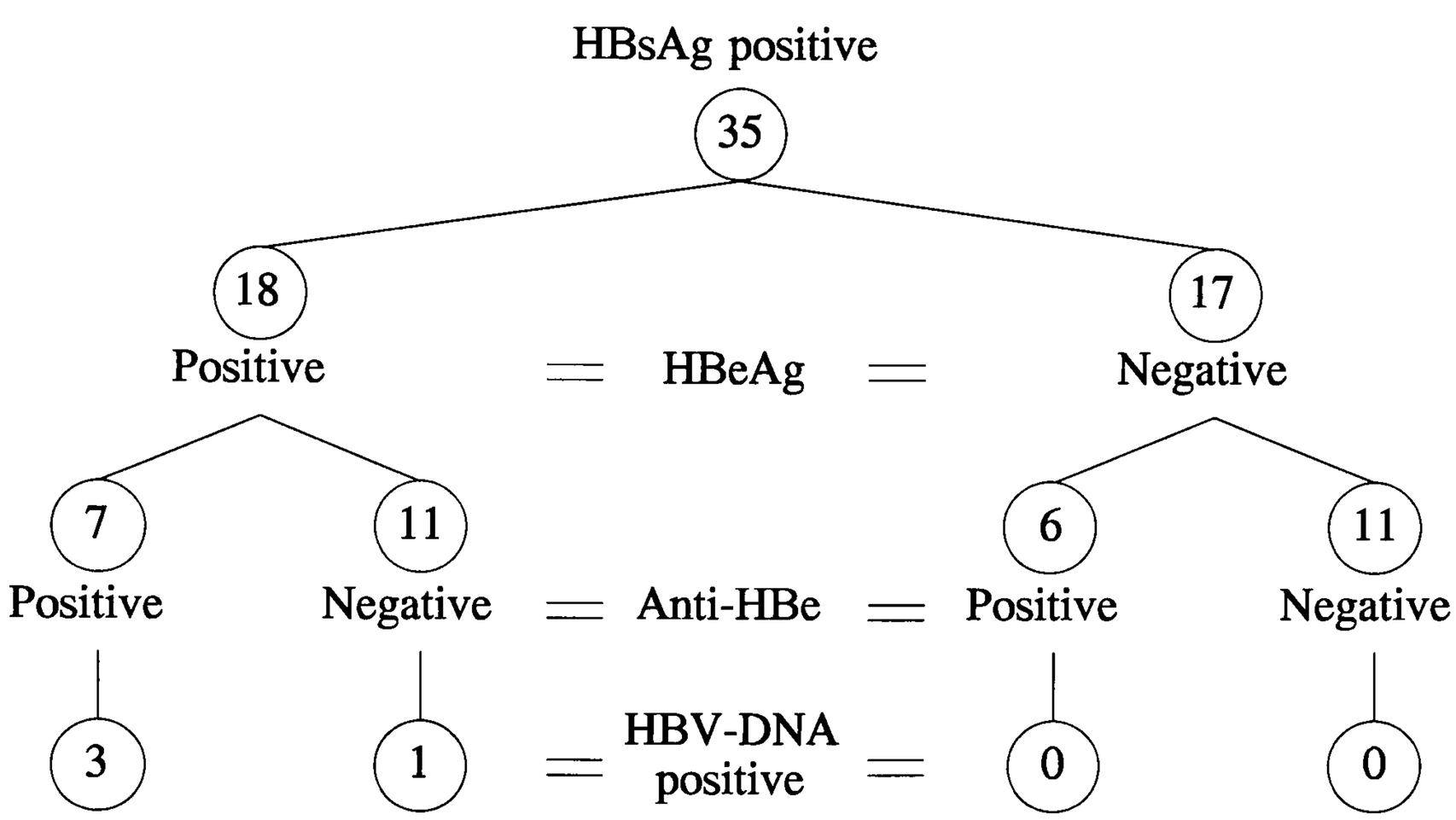


Figure 5.2 - Details of hepatitis B viral markers in the 35 HBsAg-positive subjects.

Three of the HBsAg-positive subjects were among the six with clinical signs of liver disease, including the two with jaundice (Subjects 1 and 2, Table 5.1). The latter two were from Benghazi (one a manual worker, the other an office worker) and the third (Subject 4, Table 5.1) was a blood donor from Tripoli. The other three subjects with clinical signs (Subjects 3, 5 and 6) were seropositive for anti-HBs and anti-HBc.

There were no significant differences ($p > 0.1$) in the frequencies of HBsAg seropositivity between subjects of different occupations or from the different centres but subjects from Sebha had a higher rate of exposure to HBV, overall, than those from Benghazi or Tripoli (Table 5.4).

Table 5.4 Distribution of hepatitis B virus markers between subjects from the three cities.

Blood bank centre	No.	HBsAg positive	HBsAg negative		Total positive for any HBV marker
			Anti- HBc positive	Anti-HBs positive	
Benghazi	156	18 (11.6%)	32 (20.5%)	30 (19.2%)	50 (32.1%)
Sebha	30	4 (13.3%)	12 (40.0%)*	12 (40.0%)†	16 (53.3%)*
Tripoli	115	13 (11.3%)	27 (23.5%)	20 (17.4%)	40 (34.8%)
Totals	301	35 (11.6%)	71 (23.6%)	63 (20.9%)	106 (35.2%)

* Significantly ($p < 0.05$) higher frequency than in subjects from Benghazi but not significantly different ($0.1 < p > 0.05$) from those from Tripoli.
† Significantly higher frequency than in subjects both from Benghazi ($p < 0.025$) and Tripoli ($p < 0.02$).

All of the 35 HBsAg positive subjects had abnormal serum aminotransferases, with 13 (including the four HBV-DNA positive cases) having ALT and/or AST activities > twice the upper normal limits (Fig. 5.3). Elevated ALT and AST activities > twice upper normal limit were found in 32 of the 63 anti-HBs positive subjects and in four of the eight who tested positive for anti-HBc alone.

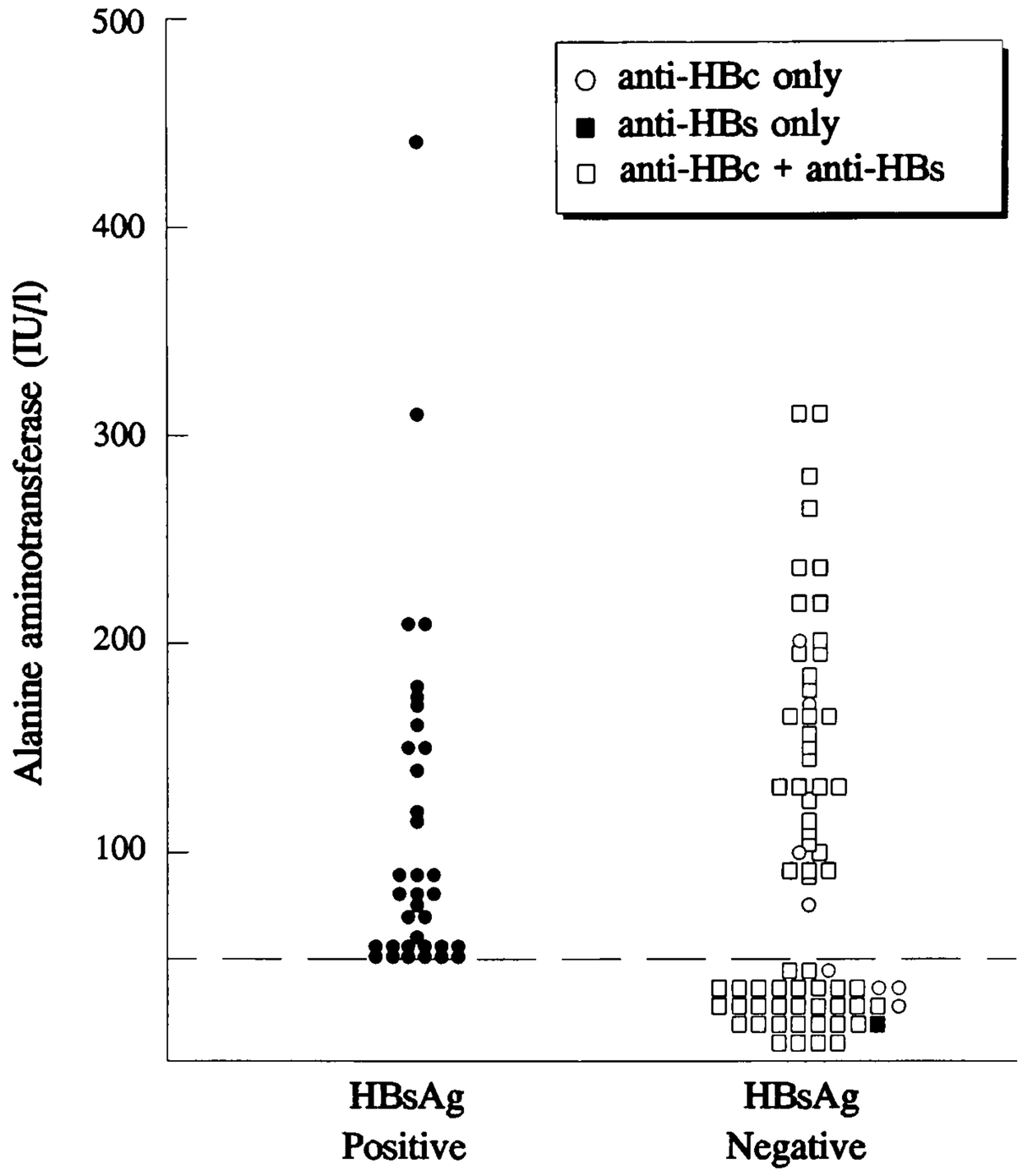


Figure 5.3 Alanine aminotransferase values in relation to HBV marker status in the 106 subjects with HBV markers.

5.1.3 Results of Hepatitis C Screening

Eighteen (6%) of the 301 subjects were found to be anti-HCV positive on initial screening. These comprised five blood donors, three students, three hospital workers and seven outpatients, and included the three HBsAg-negative subjects with clinical signs of liver disease (Sections 5.1.1 and 5.1.2). Sixteen (88.9%) of the 18 were positive for antibodies against at least one of the individual HCV peptides (Table 5.6), with 15 having antibodies against core, 11 against envelope, and 10 each against NS1, NS3/NS4 and NS5. Two subjects did not react against any of the five peptides despite being seropositive in the screening assay. Twelve (66.6%) of the 18 had circulating HCV-RNA, including three of the five anti-HCV positive blood donors. All 18 were seronegative for HBsAg, but seven were positive for anti-HBc and five for anti-HBs.

Table 5.5 **Profiles of serum antibodies against individual HCV peptides and of HCV-RNA in the 18 subjects who were seropositive for anti-HCV in the screening assay.**

No.	Age (y)	Sex	Antibodies against the different HCV peptides indicated					HCV-RNA
			core	env	NS1	NS3/4	NS5	
1	23	M	+	+	0	0	0	0
2	24	F	+	0	+	+	0	+
3	25	M	+	+	+	0	+	+
4	27	F	0	0	0	0	0	0
5	33	M	+	+	+	0	+	0
6	34	F	+	0	0	0	0	0
7	35	F	+	+	+	+	+	+
8	35	M	+	0	+	+	+	+
9	35	F	+	0	0	0	0	0
10	39	F	+	0	+	+	0	+
11	43	F	+	+	0	+	+	+
12	48	F	+	+	+	+	+	+
13	51	M	+	+	+	+	+	+
14	55	M	+	+	0	0	+	+
15	56	M	+	+	+	+	0	+
16	58	M	0	+	0	+	+	0
17	58	M	+	+	+	+	+	+
18	65	M	0	0	0	0	0	+
		TOTALS	15	11	10	10	10	12

Fourteen of the 18 anti-HCV positive subjects had elevated serum aminotransferase activities (means±SD ALT = 192±95, AST = 186±67). The four subjects with normal AST and ALT values included the three HCV-RNA positive blood donors (Table 5.6). Together with the 35 HBsAg-positive subjects, these 14 accounted for 49 of the 62 control subjects with abnormal biochemical liver tests (Section 5.1.1). The remaining 13, who were seronegative for HBsAg and anti-HCV but positive for anti-HBs and anti-HBc, were tested for HCV-RNA by PCR and three were found to have circulating HCV-RNA. In total, therefore, 21 (7.0%) of the 301 control subjects were found to be seropositive for either anti-HCV or HCV-RNA, or both.

Table 5.6 **Frequency of hepatitis C viral (HCV) markers in the different groups of control subjects.**

	No.	Anti-HCV positive		Anti-HCV negative *	Total positive for any HCV marker
		HCV-RNA positive	HCV-RNA negative	HCV-RNA positive	
Blood donors	76	3	2	0	5 (6.6%)
Hospital workers:					
Office	35	1	1	0	2 (5.7%)
Manual	34	1	0	0	1 (2.9%)
Medical students	82	2	1	1	4 (4.9%)
Outpatients	74	5	2	2	9 (12.2%)
Totals	301	12	6	3	21 (7.0%)

* Only the 13 patients with elevated serum aminotransferases were tested for HCV-RNA

The highest frequency (12.2%) of seropositivity for HCV markers was found in the outpatients but this was not statistically significantly different ($p > 0.2$) from the frequencies in the other groups. The frequency of seropositivity for both anti-HCV and HCV-RNA increased steadily with age (to 12.8% and 10.3% for anti-HCV and HCV-RNA respectively) with none of the 49 subjects under the age of 20 years being found positive (Fig. 5.4), but there was no statistically significant difference between the sexes, nor in the prevalence of HCV markers between subjects from the three cities (Table 5.7)

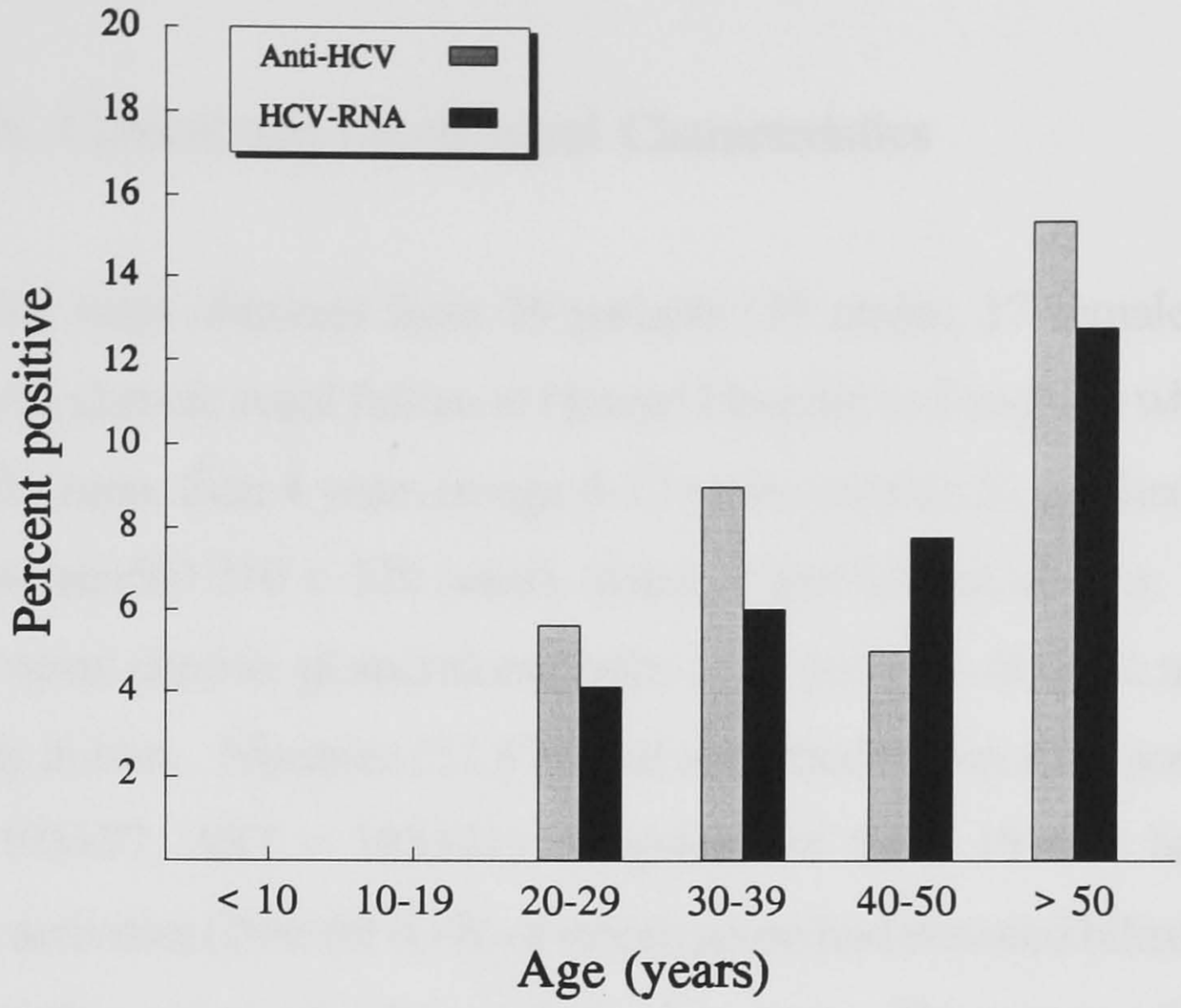


Figure 5.4 **Age-related prevalence of anti-HCV and HCV-RNA in the 266 HBsAg-negative control subjects.**

Table 5.7 **Details of hepatitis C virus markers in control subjects from the three cities.**

Blood bank centre	No.	Anti-HCV positive	HCV-RNA positive	Total positive for any HCV marker
Benghazi	156	10 (6.4%)	13	13 (8.3%)
Sebha	30	1 (3.3%)	1	1 (3.3%)
Tripoli	115	7 (6.1%)	1	7 (6.1%)
Totals	301	18 (6.0%)	15 (5.0%)	21 (7.0%)

5.2 HAEMODIALYSIS PATIENTS

5.2.1 Demographic, Clinical and Biochemical Characteristics

Serum samples were obtained from 36 patients (19 males, 17 females; median age 45, range 25-75 years) with chronic renal failure at Hawari Hospital in Benghazi who had been treated with haemodialysis for more than 4 years (range 4-12 years, median 8) and had received multiple blood transfusions (mean±SD 210 ± 120 units), mainly from Libyan donors. The causes of the chronic renal failure were: chronic glomerulonephritis in 25 patients, diabetic nephropathy in nine and polycystic kidney in two. Nineteen (52.8%) had abnormal serum ALT and/or AST activities (mean±SD ALT = 193±77, AST = 183±81). Eighteen of these 19 also had elevated serum alkaline phosphatase activities (204±69 IU/l), of whom seven had elevated bilirubin concentrations (33±8), and prothrombin time was also prolonged in two. The seven with elevated serum bilirubin were clinically jaundiced, with hepatomegaly in six and splenomegaly in three. Hepatomegaly was found in two (with splenomegaly in one) other patients with normal biochemical liver tests. None of the patients had ascites or any cutaneous stigmata of liver disease.

5.2.2 Results of HBV Screening in Haemodialysis Patients

Thirty (83.3%) of the 36 patients were seropositive for anti-HBc antibodies (Fig.5.5). This was a significantly higher apparent rate of exposure to the virus than in the control subjects (X^2= 28.96, p< 0.0001). There was also a significantly higher (p < 0.001) rate of chronic HBV infection in the renal dialysis patients, with 12 (40%) of the 36 cases being seropositive for HBsAg. None of these 12 was seropositive for HBeAg but seven had anti-HBe antibodies, of whom three were positive for serum HBV-DNA (at 5, 24 and 36 pg/ml). Of the 18 HBsAg-negative patients with anti-HBc, nine were positive for anti-HBs (Fig.5.5) but all were negative for anti-HBe and HBV-DNA. None of the patients had circulating antibodies against the hepatitis Delta virus (HDV). Thirteen of the 19 patients with abnormal biochemical liver tests had markers of exposure to HBV, including the 12 who were HBsAg seropositive (Table 5.8).

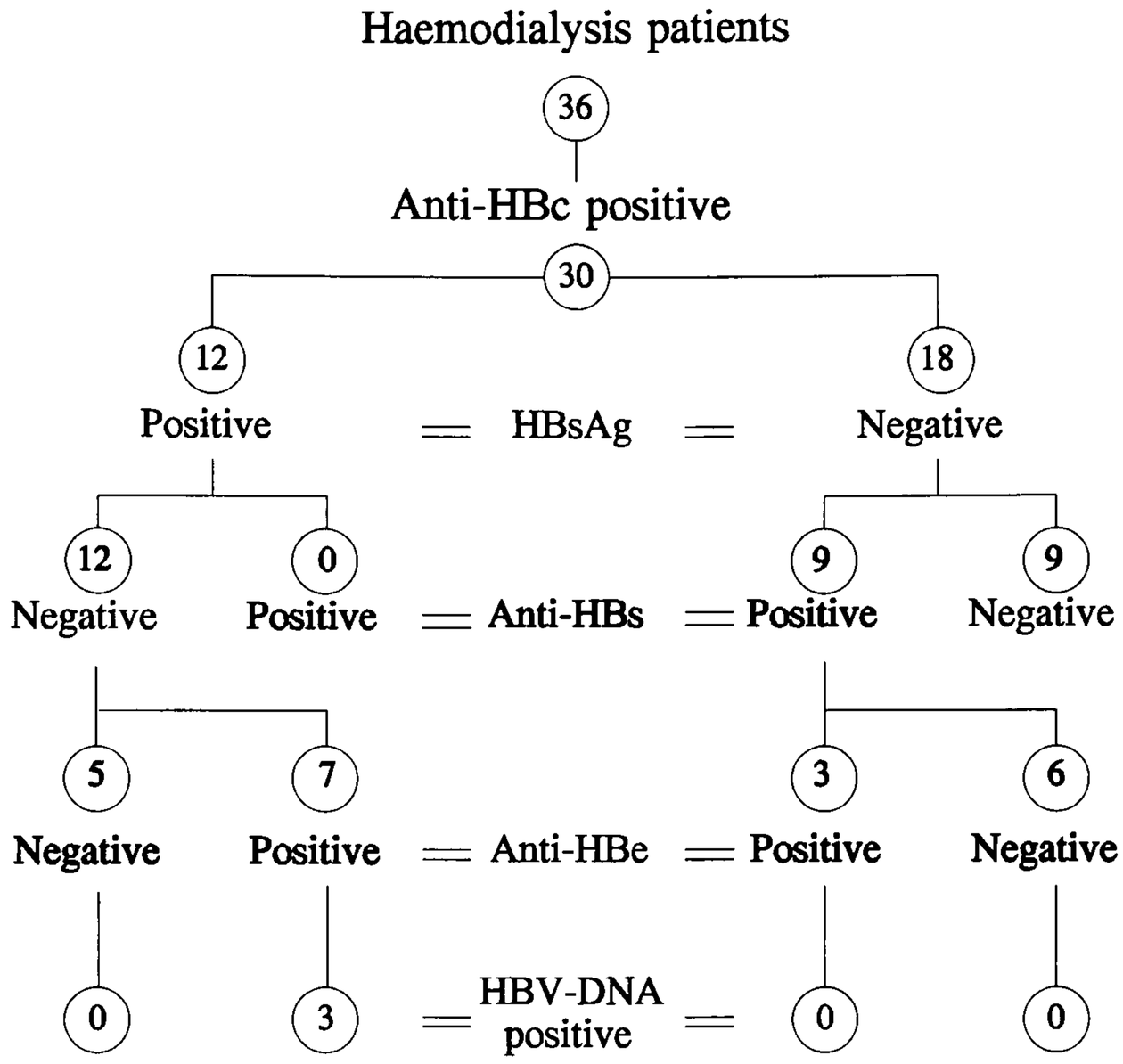


Figure 5.5 Details of hepatitis B virus serum markers in the 36 haemodialysis patients

Table 5.8 Details of the 13 haemodialysis patients with abnormal biochemical liver tests who had serum markers of HBV exposure

	Patient number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Age	25	38	45	26	39	49	52	31	62	42	55	75	48
Sex	M	F	M	F	F	M	M	F	M	F	M	M	F
Jaundice	+	+	+	+	+	+	+	-	-	-	-	-	-
Hepatomegaly	+	+	+	+	-	-	-	-	-	-	-	-	-
Splenomegaly	+	+	+	-	-	-	-	-	-	-	-	-	-
ALT *	80	90	130	210	190	290	350	160	170	190	250	130	150
AST *	65	75	110	260	240	150	180	290	210	230	180	90	310
Bilirubin *	25	31	41	28	45	34	25	18	10	6	13	9	12
Alk. phos.*	135	180	145	210	235	165	178	198	324	126	180	210	360
Anti-HBc	+	+	+	+	+	+	+	+	+	+	+	+	+
HBsAg	+	+	+	+	+	+	+	+	+	+	+	+	-
Anti-HBe	+	+	+	+	+	+	-	-	-	-	-	-	-
Anti-HBs	+	+	+	+	+	+	+	+	+	-	-	-	-
HBV-DNA	+	-	-	-	+	+	-	-	-	-	-	-	-

* Normal upper limits were 50 IU/l for ALT and AST, 120 IU/l for alkaline phospatase, and 17 μmol/l for bilirubin.

5.2.3 Results of HCV Screening in Haemodialysis Patients

Six (16.7%) of the 36 haemodialysis patients were positive for anti-HCV by the screening assay. This was a significantly higher apparent rate of exposure to the virus than in the control subjects ($X^2 = 4.05$ $p < 0.05$). All six were found to be positive for HCV-RNA by PCR and for antibodies against at least two of the individual HCV peptides (Table 5.9). None of the patients was jaundiced and all had normal serum bilirubin concentrations, but four (patients 2, 3, 4 and 6, Table 5.9) had abnormal serum ALT (260, 425, 380 and 120 IU/l, respectively) and AST (180, 385, 415 and 160 IU/l) activities. These four were all seropositive for anti-HBc but had no other markers of HBV infection. All four had hepatomegaly and one also had an enlarged spleen. The remaining two patients had normal serum biochemical liver tests and no markers of HBV infection, but one had hepatomegaly and another had splenomegaly. Overall, therefore, 32 (88.9%) of the 36 haemodialysis patients had evidence of exposure to HBV and/or HCV, with 18 (50%) having current infections with either hepatitis B (Section 5.2.2) or C viruses.

Table 5.9 HCV serology in the six anti-HCV positive haemodialysis patients

			Antibodies against the different peptides indicated					
No.	Age (y)	Sex	core	env	NS1	NS3/4	NS5	HCV-RNA
1	30	M	+	+	0	0	0	+
2	32	F	+	+	+	+	+	+
3	30	M	+	+	+	+	+	+
4	35	M	+	0	0	+	+	+
5	52	M	+	+	0	+	0	+
6	43	F	+	+	+	+	+	+
	TOTALS		6	5	3	5	4	6

5.3 PATIENTS WITH CHRONIC LIVER DISEASE

5.3.1 Demographic, Clinical and Biochemical Characteristics

Sixteen patients with chronic liver disease (9 males, 7 females; median age 45 years, range 35-62) with no history of excessive alcohol consumption or risk factors for viral hepatitis (intravenous drug use, tattooing, blood transfusion, surgery, or history of homosexuality) were recruited from the hospitals in Tripoli and Benghazi (Section 5.1.1). They had all tested negative for HBsAg by haemagglutination (Wellcotest) and had no evidence of schistosomiasis or other parasitic infections. The working diagnosis in these cases was chronic non-A, non-B viral hepatitis. All had had abnormal biochemical liver tests together with one or more of the following signs and symptoms for more than 6 months (in the absence of raised jugular venous pressure): jaundice, ascites, hepatomegaly, splenomegaly, spider angiomata, and palmar erythema (Table 5.11).

At the time of sampling, all 16 had elevated serum ALT activities (mean±SD = 329±124 IU/l), and 15 also had raised AST activities (450±180 IU/l), with abnormal prothrombin times (> 13 sec). Fourteen had raised serum bilirubin concentrations (61±50 µmol/l), with elevated alkaline phosphatase activities (245±110 IU/l) in nine. Twelve had abnormally low serum albumin concentrations (25.0±4.0 g/l). However, none of the patients had a flapping tremor, or other evidence of incipient or overt hepatic encephalopathy.

Liver biopsies were available for all patients and were reviewed for the present study (Chapter 3, Section 3.2.8). Six patients were classified as having inactive disease, with minimal portal inflammation and at most only occasional foci of necrosis with mild fibrosis and well preserved architecture (Table 5.10). Seven patients had moderate fibrosis: five with mild and two with moderate necroinflammatory activity. The remaining three patients had cirrhosis, one inactive and two with severe periportal hepatitis.

Table 5.10 Clinical, biochemical and histological data on the 16 patients with chronic liver disease

	Histological activity *				Total
	Inactive	Mild	Moderate	Severe	
M/F	4/3	3/2	1/1	1/1	16
Jaundice	5	4	2	2	13
Hepatomegaly	6	5	2	2	15
Splenomegaly	-	1	1	2	4
Ascites	-	2	2	2	6
Spider angiomata	-	-	-	1	1
Palmar erythema	-	-	-	2	2
Abnormal:					
ALT	7	5	2	2	16
AST	7	4	2	2	15
Bilirubin	6	4	2	2	14
Alkaline phosphatase	3	4	1	1	9
Prothrombin time	7	4	2	2	15
Fibrosis:					13
Mild	6	-	-	-	
Moderate	-	5	2	-	
Cirrhosis	1	-	-	2	3

* Necroinflammatory activity as defined in Chapter 3 (Section 3.2.8)

5.3.2 Results of Screening Chronic Liver Disease Patients for Hepatitis B and C.

Ten of the 16 patients were found to be seropositive for anti-HBc, four of whom were HBsAg-positive and the remaining six all had anti-HBs. All were seronegative for anti-HBe antibodies. None had evidence of active viral replication, as indicated by seronegativity for both HBV-DNA and HBe-Ag, and none had anti-HDV antibodies.

Ten patients were seropositive for anti-HCV on initial screening and all 10 were also positive for HCV-RNA by PCR. All 10 also had antibodies against at least one of the

individual HCV peptides (Table 5.11). None of these 10 was positive for HBsAg but four had anti-HBc and anti-HBs. Overall, therefore, 14 of the 16 patients had chronic infections with HBV (HBsAg positive, 4 patients) or HCV. The remaining two patients (both anti-HBs positive) probably had chronic hepatitis B but appeared to have cleared the virus, at least from their blood.

Table 5.11 HCV serology in the ten anti-HCV positive chronic liver disease patients

No.	Age (y)	Sex	HCV Screen	Antibodies against the different HCV peptides indicated					HCV-RNA
				core	env	NS1	NS3/4	NS5	
1	56	F	+	0	+	0	+	0	+
2	46	F	+	+	0	+	+	+	+
3	62	M	+	+	+	+	0	+	+
4	35	M	+	0	0	0	+	+	+
5	48	M	+	+	0	0	+	0	+
6	43	F	+	+	+	+	+	+	+
7	40	F	+	0	+	+	0	+	+
8	37	M	+	+	0	0	0	+	+
9	49	F	+	+	0	+	+	+	+
10	38	M	+	+	+	0	0	0	+
TOTALS			10	7	5	5	6	7	10

* Patients positive for anti-HBc and anti-HBs

5.4 SCREENING FOR HEPATITIS GBV-C VIRUS IN LIBYAN SUBJECTS

Sera from all of the 74 control subjects who had elevated serum aminotransferases (Table 5.2), all of the 36 haemodialysis patients and the 16 patients with chronic liver disease were tested by PCR for evidence of infection with the GBV-C virus (Chapter 3, Section 3.2.6). None of the control subjects or patients with chronic liver disease were found to be positive. GBV-C genomic material was, however, detected in sera from four of the haemodialysis patients. Two of these (patients 2 and 4, Table 5.9) had chronic HCV infection. The other two were seronegative for HBsAg and anti-HCV, and had normal biochemical liver tests with no clinical evidence of liver disease.

5.5 DISCUSSION

The above results indicate that there is a high rate of exposure to the hepatitis B and C viruses among the Libyan population at large. Overall, 112 (37.2%) of the 301 control subjects had at least one serological marker of HBV or HCV. The proportion exposed to HCV may, however, be an underestimate because testing for HCV-RNA in the present study was confined to serum of subjects who were anti-HCV positive and/or had abnormal biochemical liver tests. There is evidence that HCV infected individuals may remain seronegative for anti-HCV and HCV-RNA while harbouring the virus in their livers or peripheral blood mononuclear cells (Pereira et al. 1995; Schmidt et al. 1995), and that up to 20% of anti-HCV negative blood donors with normal serum aminotransferases whose blood can be implicated in post-transfusion hepatitis may be HCV-RNA seropositive (Villa et al. 1991).

Significantly, seven of the professedly healthy blood donors were HBV carriers (HBsAg seropositive) and a further three had current HCV infection (HCV-RNA positive). Thus, 13.2% of the blood donor population was actively infected with one or other of these viruses. This might account for the very high incidence of exposure to HBV and HCV among the haemodialysis patients studied here, with 30 of the 36 patients having at least one HBV marker and a further two having chronic HCV infection without HBV markers, i.e. a total of 88.9% who had been exposed to either or both viruses.

For economic reasons screening of donated blood in Libya has involved testing only for HBsAg and only by using the Wellcotest haemagglutination assay. The present findings indicate that this approach is inadequate for eliminating the risk of transmission of HBV via blood transfusion. Had serum aminotransferase activities been used as a surrogate marker, the seven HBsAg-positive blood donors would have been identified, since all had elevated AST and ALT activities, but this would not have excluded the three HCV-RNA positive blood donors (all of whom had normal aminotransferases). One additional control subject who was seropositive for HCV-RNA also had normal AST and ALT values, making a total of four (22%) of the 18 anti-HCV positive subjects who had chronic HCV infection with normal biochemical liver tests. This is in keeping with previous reports that the use of surrogate markers would not detect 20-50% of blood donations involved in transmission of non-A, non-B hepatitis(Polesky and Hanson, 1989) or, more specifically, of HCV(van der Poel, 1994; Villa et al. 1991; Garson et

al. 1990a) and that up to 70% of patients with chronic hepatitis C may have persistently normal serum aminotransferases (Esteban et al. 1991; Alberti et al. 1992; McGuinness et al. 1993; Romeo et al. 1993; Shindo et al. 1995; Silini et al. 1995).

It is noteworthy also that there were ten of the control subjects with abnormal biochemical liver tests who, although seropositive for anti-HBc and anti-HBs, did not have any markers of ongoing HBV or HCV infection. All were apparently healthy with no clinical signs of liver disease. As screening for hepatitis A (HAV) and E (HEV) viruses was not included in the design of this study these might have been cases of sub-clinical infection with one or other of these viruses. On the other hand, detailed investigations to exclude other liver diseases such as autoimmune hepatitis, Wilson's disease and haemochromatosis were not undertaken and it is possible that they may have been asymptomatic cases of some such disorder.

The high rate of exposure to HBV (35.2%) and high carriage rate of the virus (11.6% HBsAg-positive) observed in the present study is in keeping with reports of the prevalence of HBV in areas with intermediate-high endemicity (Chapter 1, Section 1.3.3) and is comparable to the HBsAg positive rate of 16.4% recently reported for Egypt (Waked et al. 1995). Interestingly, none of the control subjects or patients studied was found to have circulating antibodies against the delta virus (HDV), which is in contrast with the frequencies of 6-27% anti-HDV seropositivity reported for populations studied in Ethiopia and Cameroon (Rapicetta et al. 1988; Ndumbe, 1991). The reason for this difference is unclear, but there is very little intravenous drug abuse in Libya (see Chapter 1, Section 1.3.3). However, it indicates that failure to detect HBV-DNA in nine of the HBsAg positive subjects in the present study was not due to suppression of HBV replication by HDV (Chapter 1, Sections 1.3.2 and 1.3.4).

The frequency of exposure to HCV in the normal Libyan population (7.0%) documented here is more than twice that (3.0%) which has been reported (Coursaget et al. 1990a) for the neighbouring country of Tunisia to the north-west (Fig. 5.1) but much lower than that (14-24%) reported for Libya's eastern neighbour, Egypt(Darwish et al. 1992; Kamel et al. 1992; Waked et al. 1995), and is more in line with the prevalence (6.4-14.5%) of HCV found in other African countries (Darwish et al. 1993; Ngatchu et al. 1992; Delaporte et al. 1993; Mets et al. 1993; Waked et al. 1995). Among the control subjects in the present study, no significant difference was noted in the frequency of anti-HCV or HCV-RNA positivity between blood donors and

hospital workers or medical students, who might have been considered more "at risk". Since none of these subjects had any other known risk factors for viral hepatitis, all of the cases of HCV exposure documented in the control subjects therefore seem to be instances of "sporadic" infection (Chapter 1, Section 1.4.2). It is noteworthy also, that these sporadic infections appear to be acquired either many years ago or later in life, since none of the 49 control subjects in the present study who were under the age of 20 years was found to be anti-HCV positive. This is in agreement with other reports that the seroprevalence of HCV markers increases with age (Stevens et al. 1990).

Screening for antibodies against the individual HCV peptides provided little additional information over and above that acquired from results of the anti-HCV screening assay and PCR for HCV-RNA. All but two of the 34 control subjects and patients who were anti-HCV positive by the screening assay also had antibodies reacting with at least one of the five HCV peptides tested and 30 reacted with two or more. Antibodies against the HCV core peptide were found most frequently (in 28 of the 34) and anti-NS1 antibodies least frequently (18/34), but there was no particular correlation between the various patterns of reactivity and any of the parameters investigated. Of the two subjects (both in the control group) whose sera gave positive reactions in the anti-HCV screening assay but did not react against any of the peptides, one was HCV-RNA seropositive and the other was seronegative. In this latter patient, positivity in the screening assay may have been a false-postive result. Alternatively she may have had a previous acute (or subacute) infection, for anti-HCV antibodies have been shown to persist for six to 12 months after recovery in the majority (90%) of patients with acute hepatitis C and for up to 10 years in about 15% (Dittmann et al. 1991; Barrera et al. 1995). This might also be the case in the other five anti-HCV positive control subjects who were HCV-RNA seronegative. However, interpretation of these results is very difficult because the viraemia in asymptomatic infections may fluctuate (Romeo et al. 1993); Bruno et al, 1994; (Myhre et al. 1994; Barrera et al. 1995; Blajchman et al. 1995) and, as noted above, patients can be seronegative for HCV-RNA while still harbouring the virus in their livers or at extrahepatic sites (Koskinas et al. 1995; Saleh et al. 1994b; Pereira et al. 1995; Schmidt et al. 1995).

It is interesting to note that in the present study chronic HBV and HCV infections appeared to be mutually exclusive, in that although 24 of the 37 control subjects and patients with evidence of HCV exposure (anti-HCV or/and HCV-RNA positive) had anti-HBc and 12 had

anti-HBs antibodies, none was HBsAg seropositive. HBV/HCV co-infections have been widely reported in patients with chronic liver disease (Fong et al. 1991; Porchon et al. 1992; Liaw et al. 1994; Villa et al. 1995) or hepatocellular carcinoma (Paterlini et al. 1993; Shiratori et al. 1995), and such patients tend to have greater severity of liver disease than those infected with only one of these viruses. However, considering that some of the risk factors for acquisition are similar for both viruses, overall such co-infections seem to be relatively rare (about 10-15%) (Porchon et al. 1992; Esteban, 1993; Liaw, 1995). Earlier studies in chimpanzees with chronic HBV had shown that superinfection with non-A, non-B (presumably C) virus was accompanied by a marked reduction in serum HBsAg titres (Bradley et al. 1983). This has since been confirmed in chronic hepatitis B patients with acute HCV superinfections. In such cases, there is a gradual decrease in serum HBV-DNA with eventual HBeAg/anti-HBe seroconversion and, in some cases, eventual disappearance of HBsAg (Liaw et al. 1994; Liaw et al. 1991; Pontisso et al. 1993);. It has been suggested that this may be due to suppresion of HBV replication by HCV (Fong et al. 1991; Liaw et al. 1994). The mechanisms by which HCV may achieve this are not yet clear but a cotransfection study demonstrating suppression of HBV replication by HCV in a human hepatoma (Huh-7) cell line has suggested that the processes of transcription and encapsidation of HBV pregenomic RNA are involved and that the HCV core protein may have a gene-regulatory function (Shih et al. 1993). Unforunately, it is impossible to say whether these considerations apply in the present study because the various groups of subjects were investigated at only one time point and whether some were cases of HCV superinfection on a background of chronic hepatitis B, with HCV-induced clearance of HBV, cannot be determined.

Finally, it is also interesting to note that GB virus infection (at least in so far as reflected in seropositivity for GBV-C genomic material) seems to be very rare in the Libyan population. Of the 126 sera tested, only four (3.2%) were positive for GBV-C by PCR and these four were all multiply transfused haemodialysis patients. This contrasts with findings elsewhere in Africa that nearly 30% of subjects tested had evidence of exposure to the GBV viruses on the basis of seropositivity for anti-GBV antibodies(Zuckerman, 1995).

CHAPTER 6

HEPATITIS C VIRUS GENOTYPES

IN LIBYA AND ELSEWHERE

6.1 SUBJECTS

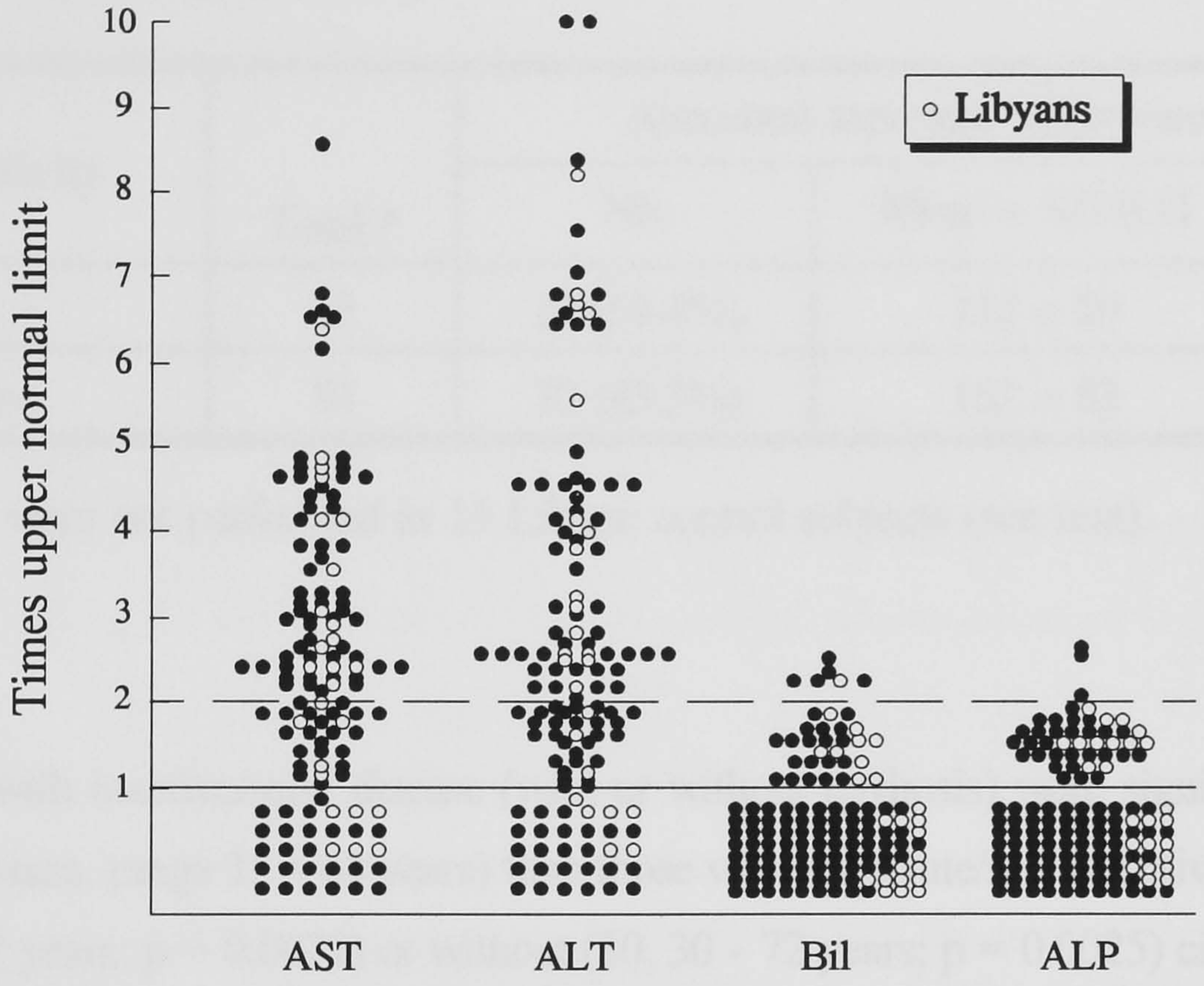
Genotyping of the hepatitis C virus was performed in 25 of the 31 Libyan subjects who had been found to be seropositive for HCV-RNA. These comprised the 15 control subjects and the 10 who had been hospitalised with chronic liver disease who were found to be carrying the virus (Chapter 5). The study was confined to these 25 because none had had any risk factors for acquiring viral hepatitis and it was felt that their genotypes would be more representative of the population at large than might be the case with the multiply transfused haemodialysis patients. Genotypes were compared with those in a consecutive series of 106 patients with chronic hepatitis C from other parts of the world attending the Institute of Liver Studies, who were typed concurrently before institution of any anti-viral therapy. For purposes of analysis, patients were grouped according to geographical origin as shown in Table 6.1.

Table 6.1 Demographic details of subjects studied

Group	Country of Origin	Number	M/F	Age (years)		Total in Group
				Range	Median	
1 (Libya)	Libya	25	14/11	24 - 65	43	25
2 (Egypt)	Egypt	32	26/6	38 - 64	50	32
3 (Middle East)	Bahrain	2	1/1	40/45	40/45	
	Iran	1	1/0	30	30	
	Jordan	3	1/2	15 - 49	47	
	Saudi Arabia	2	2/0	51/71	51/71	
	Oman	1	0/1	34	34	
	Kuwait	3	1/2	51 - 64	59	
	UAE	5	4/1	31 - 56	44	
	Yemen	2	2/0	55/55	55	19
4 (U.K.)	England	25	17/8	30 - 66	42	
	Scotland	2	2/0	30/34	30/34	27
5 (Southern Europe)	Italy	5	2/3	35 - 53	45	
	Portugal	1	0/1	43	43	
	Spain	2	1/1	29/51	29/51	8
6 (Sub-Saharan Africa)	Gambia	1	1/0	45	45	
	Nigeria	2	2/0	49/52	49/52	
	South Africa	1	0/1	61	61	
	Senegal	1	1/0	39	39	5
7 (Asia)	India	4	1/3	36 - 61	45	
	Pakistan	6	4/2	33 - 72	46	10
8 (Caribbean Area)	Guayana	1	0/1	43	43	
	Jamaica	3	3/0	35 - 47	44	
	Venezuela	1	1/0	61	61	5
	Totals	131	87/44	15 - 72	46	131

6.1.1 Biochemical and Histological Data

Serum biochemical liver tests were performed in all 131 subjects. Serum aspartate (AST) and alanine (ALT) aminotransferase activities varied widely: from normal (in 31 subjects) to ten times the upper normal limit (Fig. 6.1). Marked elevations (> 2 x upper normal limit) in serum bilirubin concentrations and/or alkaline phosphatase activities were seen in only six subjects.



Fi gure 6.1 **Serum biochemical liver tests in the 131 subjects studied.**
AST = aspartate aminotransferase; ALT = alanine aminotransferase; Bil = bilirubin; ALP = alkaline phosphatase. Upper normal limits were: AST and ALT 50 IU/l, Bil 20 μmol/l, and ALP 120 IU/l.

Histological data from liver biopsies were available for all except the 15 Libyan control subjects (above). On the basis of the histological findings, patients were grouped according to whether they had inactive/mild or moderate/severe disease as described in Chapter 3 (Section 3.2.8).

Within each histological group there was no correlation between serum biochemical abnormalities and the presence or absence of cirrhosis, but patients with inactive/mild disease had abnormal serum aminotransferase activities significantly less frequently than those with moderate/severe disease (19 of 32 = 59.4% vs. 70 of 84 = 83.3%, respectively; $p = 0.013$). However, in patients with abnormal serum aminotransferases, there was no significant correlation with histological disease

activity as shown for example for AST values in Table 6.2. Histological assessment was therefore felt to be a more reliable index of necroinflammatory activity than serum biochemical liver test results and was used for all further analyses of the data.

Table 6.2 Comparison of serum aspartate aminotransferase activities with histologically assessed disease activity.

Histological activity	Total *	Abnormal aspartate aminotransferase		
		No.	Mean ± SD IU/l	P
Inactive/mild	32	19 (59.4%)	132 ± 89	0.163
Moderate/severe	84	70 (83.3%)	162 ± 83	

* Liver biopsies were not performed in 15 Libyan control subjects (see text).

Patients with inactive/mild disease (with or without cirrhosis) were significantly younger (median age 40 years, range 15 - 64 years) than those with moderate/severe activity with (median 48, range 30 - 71 years; p = 0.0022) or without (50, 30 - 72 years; p = 0.0025) cirrhosis. The age difference between patients with and without cirrhosis who had moderate/severe activity was not significant (p = 0.748). Males tended to have moderate/severe disease more frequently (60 of 78 = 76.9%) than females (24 of 38 = 63.2%) but this difference was not statistically significant (p = 0.182).

In 75 cases (57.3%) no obvious risk factors for acquisition of hepatitis C virus infection could be identified and these were presumed to be instances of "community-acquired" or "sporadic" infection (Table 6.3). Of the remainder, presumed routes of infection were: via blood transfusion in 33 cases (25.2%), intravenous drug use in 17 (13.0%), and occupational exposure (in medical or paramedical workers) in six (4.6%). There was no correlation between histological severity of liver disease and the presumed route of acquistion of the virus (Table 6.3): moderate/severe disease on liver biopsy being found in 72.7%, 70.6%, 66.7% and 73.3% of those in which acquisition was related to blood transfusion, intravenous drug use, occupational exposure, or "sporadic" infection, respectively.

Table 6.3 **Distribution of subjects according to mode of acquisition of hepatitis C virus infection and histological severity of liver disease**

Group	Numbers of subjects with feature indicated					
	Mode of acquisition				Liver histology	
	BT	IVDU	Occup.	Unknown	Inactive/mild	Moderate/severe
Libya	0	0	0	25	4*	6*
Egypt	11	0	2	19	6	26
Middle East	9	0	0	10	4	15
U.K.	9	14	3	1	9	18
Southern Europe	0	3	0	5	3	5
Sub-Saharan Africa	0	0	0	5	1	4
Asia	4	0	1	5	3	7
Caribbean Area	0	0	0	5	2	3
Totals	33	17	6	75	32	84
Liver Histology: Inactive/ mild Moderate/severe	 9 24	 5 12	 2 4	 16 44		

* Liver biopsies were performed on only 10 of the 25 Libyan subjects (see text).
BT = Blood transfusion IVDU = Intravenous drug use Occup. = Occupational exposure

6.1.2 Screening for Hepatitis GB Virus Infection in the Non-Libyan Subjects

All of the 106 non-Libyan chronic HCV patients were screened for GB virus infection by PCR for circulating GBV-C RNA (Chapter 3, Section 3.2.6). As reported in Chapter 5, none of the 25 HCV-RNA positive Libyan subjects in this phase of the study had GBV infection. By contrast, overall 36 (33.9%) of the 106 non-Libyan chronic HCV patients were seropositive for GBV-C RNA (Table 6.4), with 55% (15/27) in the U.K. group infected. The latter was due mainly to the high proportion of IVDUs among the U.K. patients tested, 12 (85.7%) of the 14 of whom were seropositive for GBV-C RNA. One of the two Italian IVDUs was also positive. The incidence was also high in the Sub-Saharan African (60%) and the Asian (50%) groups, but the numbers were quite small. Twenty-five percent of the Egyptian patients were seropositive for GBV-C RNA, while among the Middle Eastern patients only four (21%) were positive: the two Saudi Arabians and two of the five from the United Arab Emirates.

Apart from the IVDUs, the majority (15) of the other GBV-positive individuals were apparently cases of sporadic infection. Blood transfusion could be implicated in only five cases and there was one case in which HCV and GBV seem to have been acquired as a consequence of occupational exposure. It is noteworthy that 29 (80.6%) of the 36 GBV-positive cases had histologically moderate/severe disease compared with 48 (68.5%) of the 70 GBV-negative non-Libyan patients, but this tendency towards more severe disease in the GBV-infected individuals was not statistically significant ($p > 0.2$).

Table 6.4 Results of screening for GBV-C RNA in sera from non-Libyan patients.

Group	Country of origin	Number tested	GBV-C RNA seropositive		IVDUs positive *
			Number	Percent	
Egypt	Egypt	32	8	25%	-
Middle East	Bahrain	2	0	-	-
	Iran	1	0	-	-
	Jordan	3	0	-	-
	Kuwait	3	0	-	-
	Oman	1	0	-	-
	Saudi Arabia	2	2		-
	UAE	5	2	40%	-
	Yemen	2	0	-	-
U.K.	England	25	13	52%	10/12 (83.3%)
	Scotland	2	2		2/2
Southern Europe	Italy	5	1	20%	1/2 (50%)
	Portugal	1	0	-	-
	Spain	2	0	-	0/1 (0%)
Sub-Saharan Africa	Gambia	1	1		-
	Nigeria	2	2		-
	Senegal	1	0	-	-
	South Africa	1	0	-	-
Asia	India	4	2	50%	-
	Pakistan	6	3	50%	-
Caribbean Area	Guayana	1	0	-	-
	Jamaica	3	0	-	-
	Venezuela	1	0	-	-
Totals		106	36	33.9%	13/17 (76.5%)

* IVDUs - Intravenous drug users, number positive/number tested

6.2 GENOTYPING BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS

As described in Chapter 3, genotypes of the hepatitis C virus can be predicted from the

distinctive patterns of electropherotypes produced after digestion of RT-PCR products by different restriction enzymes. In the present study, RFLP analysis was performed on the RT-PCR products from each of the 131 HCV-RNA positive subjects by examining the electropherotype patterns produced after digestion with the restriction enzyme combination *Hae*III-*Rsa*I and comparing these with the patterns obtained after digestion with a second combination set of restriction enzymes, *Mva*I-*Hinf*I.

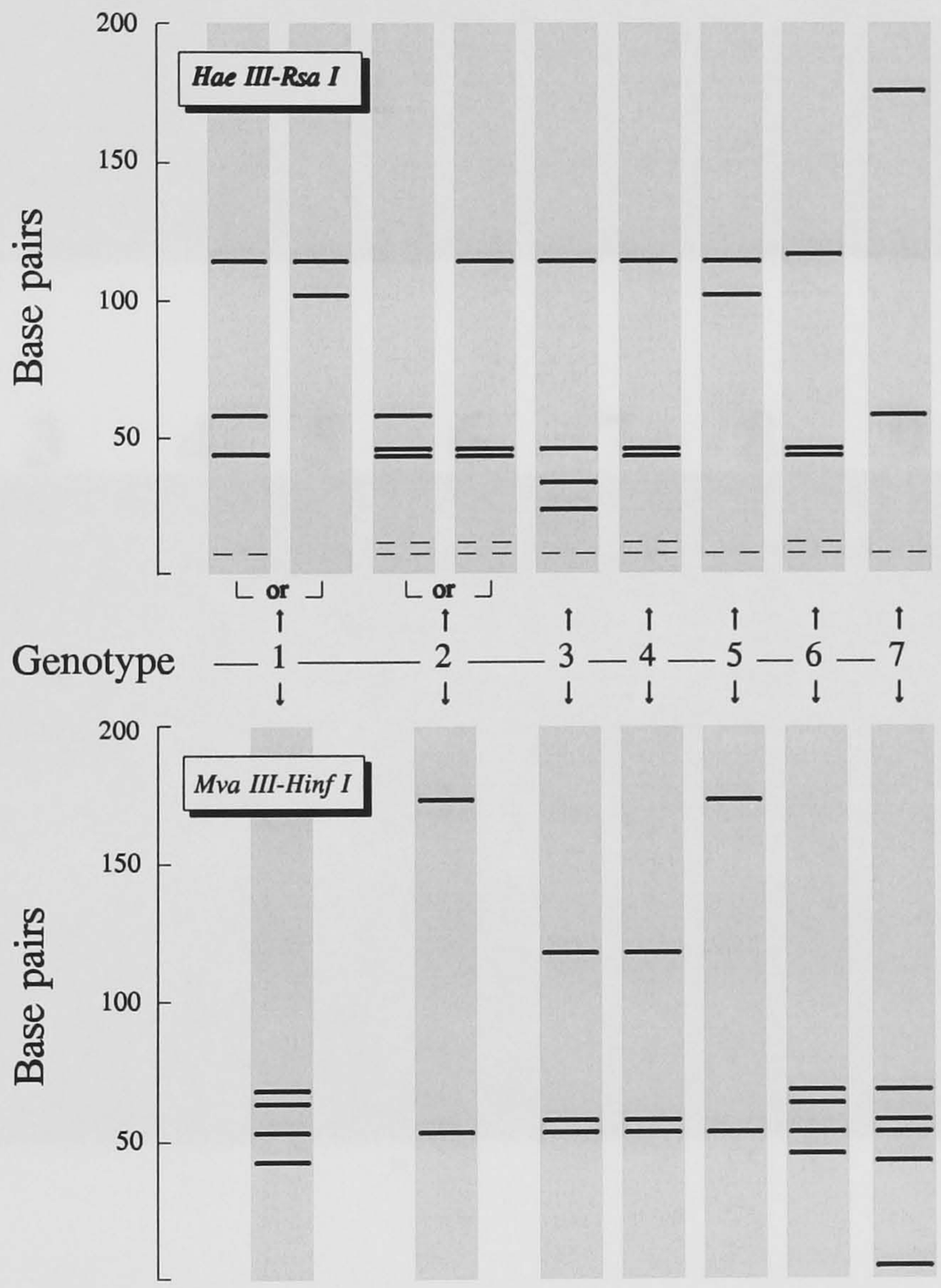
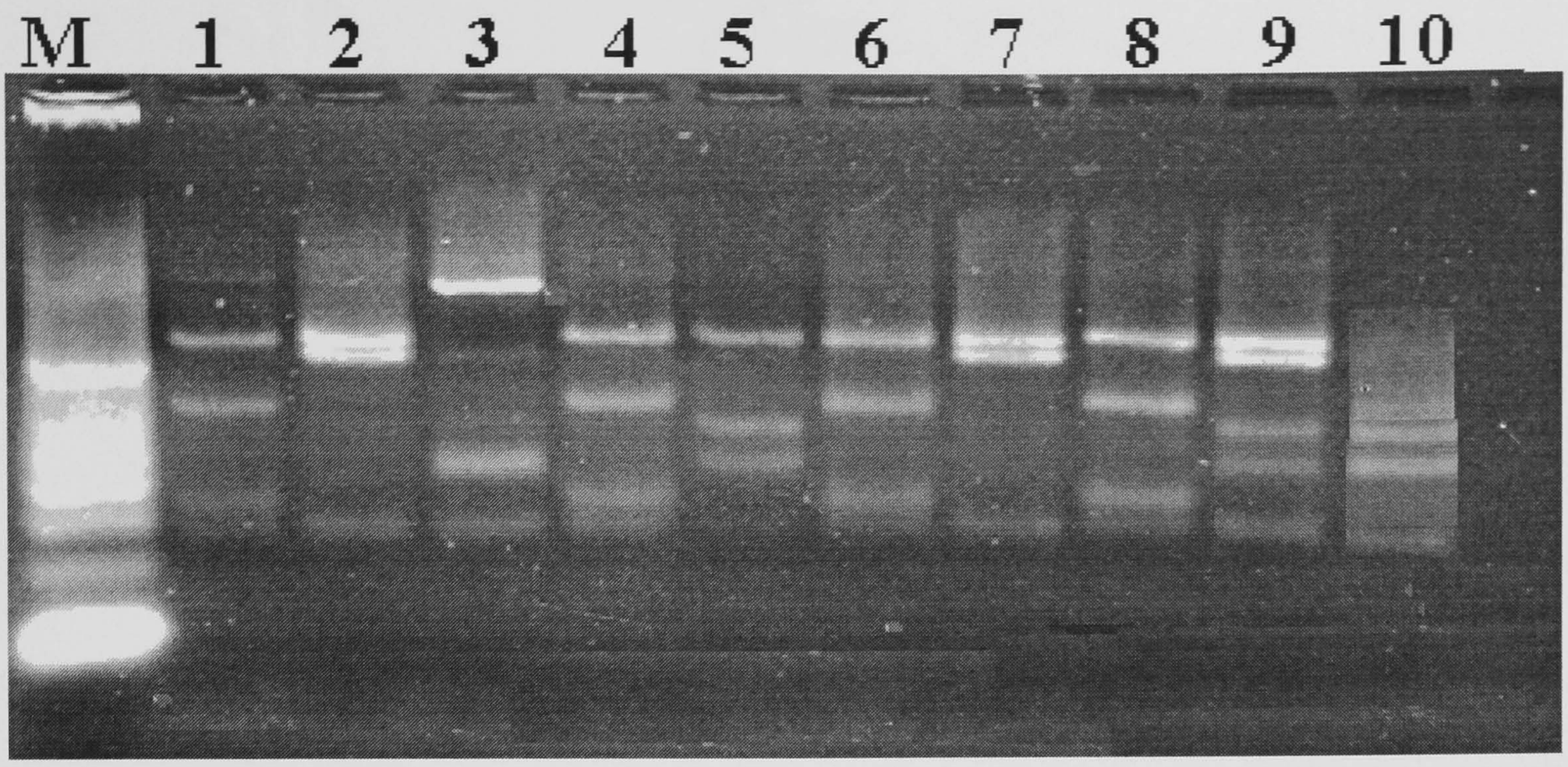


Figure 6.2 **Diagrammatic representation of the electrophoretic patterns produced by different hepatitis C virus genotypes after digestion of RT-PCR products by the restriction enzyme combinations *HaeIII-RsaI* and *MvaI-HinfI*.** For details see text.

The expected patterns produced by the different HCV genotypes after digestion with these two restriction enzyme combinations are shown diagrammatically in Fig. 6.2 and a typical example of an electrophoretic gel is shown in Fig. 6.3.

a) ***HaeIII-RsaI***



b) ***MvaI-HinfI***

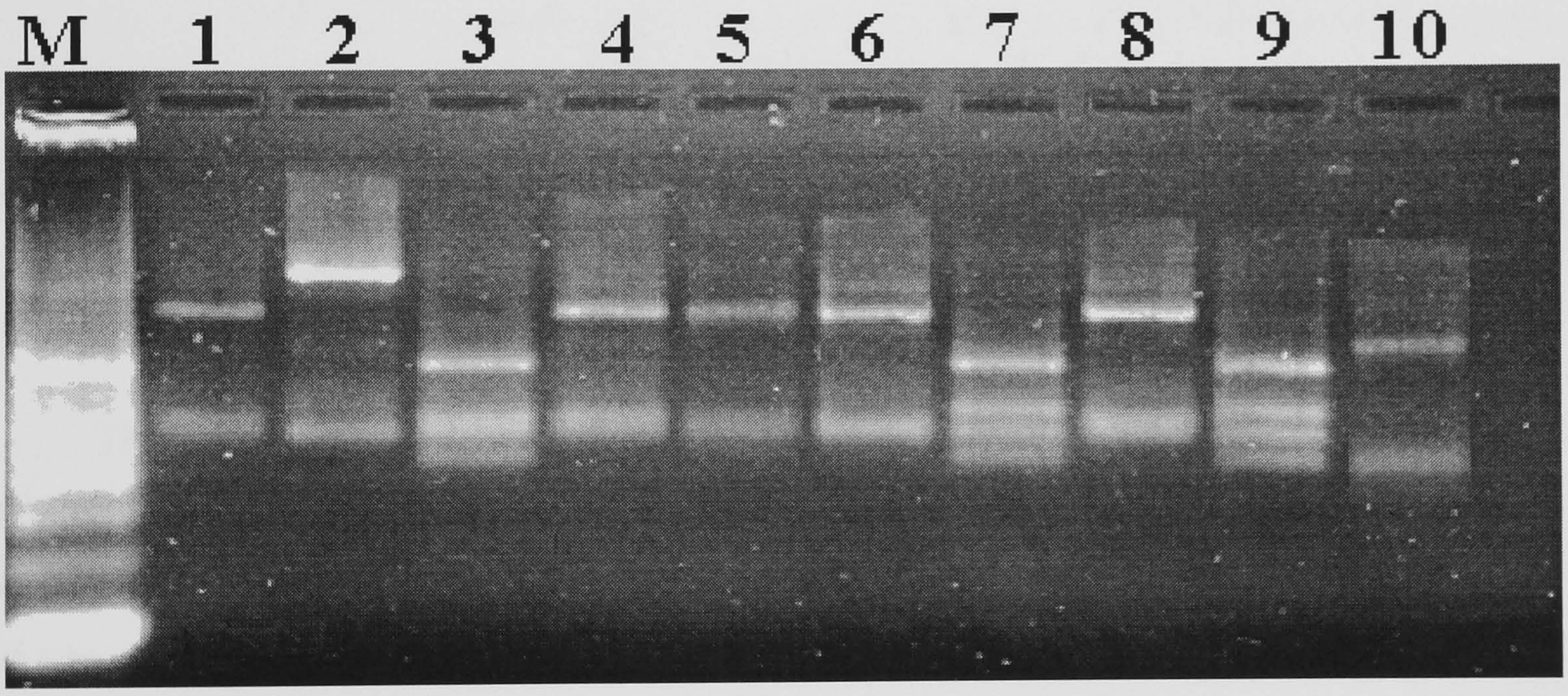


Figure 6.3 **A typical gel showing patterns of bands obtained after digestion of heptatitis C virus RT-PCR products with the restriction enzyme combinations *HaeIII-RsaI* and *MvaI-HinfI*.** [Lane 1, 4, 6, and 8: genotype 3; Lane 2: genotype 5; Lanes -3, 7, and 9: genotype 1; Lane 5: genotype 4; lane 10: genotype 2]

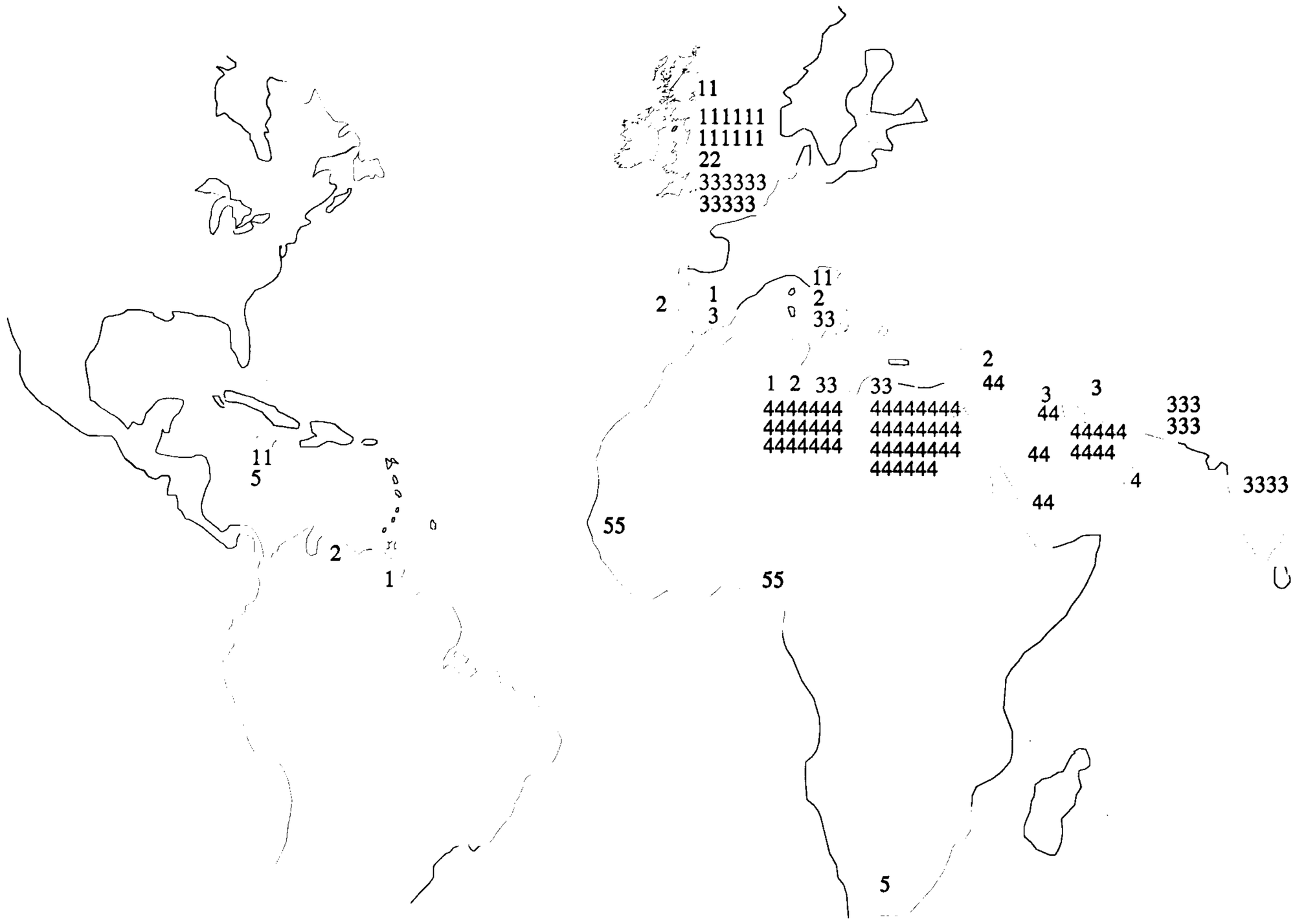


Figure 6.4 **Distribution of HCV genotypes 1 to 5 in the 131 subjects according to geographical area.** Each digit represents a single patient with the genotype indicated by the digit.

The results of the RFLP analysis showed that the subjects from North Africa were almost exclusively infected with HCV genotype 4, which was found in 21 (84.0%) of the 25 Libyans and in 30 (93.8%) of the 32 Egyptians (Fig. 6.4). Of the four Libyans with other genotypes, two had type 3 and one each had types 1 and 2, while both of the two Egyptians without type 4 had type 3. Type 4 was equally common amongst patients from the Middle East, being found in 16 (84.2%) of the 19 subjects studied from this area - two of the remaining three having type 3 and one type 2.

Genotype 4 appeared to be entirely restricted to North Africa and the Middle East, since it was not identified in any subject from any of the other areas. Among the U.K. patients, types 1 and 3 predominated - these two types being found respectively in 14 (51.9%) and 11 (40.7%) of the 27 cases studied. The two U.K. patients without these types were both from England and both had type 2. The Southern European cases represented a mixture of types 1, 2 and 3, with two of the five Italians and one of the two Spanish patients having type 1, the third Italian and

the sole Portugese patient having type 2, and the remaining two Italians and the second Spanish patient having type 3. All of the 10 patients from India and Pakistan were infected with genotype 3, while all of the five cases from Sub-Saharan Africa had type 5. Type 5 was also found in one patient (a Jamaican) from the Caribbean area. The two other Jamaicans in this group had type 1, as did the one patient from Guayana, while the one Venezualan was infected with type 2.

Examination of the distribution of genotypes in those groups in which there were sufficient numbers for statistical analysis revealed no significant associations between genotypes and the presumed routes of infection in the Egyptian, Middle Eastern and Asian patients. Thus, among the 32 Egyptian patients, 18 (60.0%) of the 30 with type 4 were cases of sporadic infection and 11 (36.7%) had a history of blood transfusion - the remaining cases being two with occupational exposure (one type 3 and one type 4) and one sporadic case with type 3 (Table 6.5). Similarly, of the 16 Middle Eastern patients with type 4, eight each had a history of blood transfusion or were cases of sporadic infection - the remaining three cases comprising two with sporadic infection (one type 2 and one type 3) and one with genotype 3 who had a history of blood transfusion. Of the 10 Asian patients (all type 3), four had a history of blood transfusion, five were sporadic cases and one had a history of occupational exposure.

Table 6.5 Analysis of genotypes with respect to presumed routes of infection

Route of infection	Number with genotype indicated					Totals
	1	2	3	4	5	
Blood transfusion	9	0	5	19	0	33
Intravenous drug use	3	0	14	0	0	17
Occupational	1	2	2	1	0	6
Unknown ("sporadic")	8	5	9	47	6	75
Totals	21	7	30	67	6	131

In contrast, in the U.K. and Southern Europe, genotype 3 was confined to intravenous drug users (IVDUs). Of the 17 IVDUs from these areas, 11 (78.6%) of the 14 from the U.K. and all of the three from Southern Europe (two Italians and one Spanish) had genotype 3. The

remaining three IVDUs (one from England and the only two patients from Scotland) all had genotype 1. Genotype 3 was not found in any other patient from these areas. Of the 13 non-IVDU patients from the U.K., nine with a history of blood transfusion had type 1, one with occupational exposure had type 1 and two other such cases had type 2, and the one case of sporadic infection had type 1. The five non-IVDU Southern European patients were all cases of sporadic infection. Three had type 1 and two had type 2.

Moderate/severe disease activity on liver biopsy was found least often in patients with genotype 2 and most frequently in those with type 5 (Table 6.6) but, perhaps because of the small numbers, this difference was not statistically significant ($p = 0.677$) and there were no significant differences ($p > 0.1$) in histological severity of disease between these and/or any of the other groups of patients infected with the different genotypes.

Table 6.6 Histological activity in relation to hepatitis C virus genotype in the 116 patients in whom liver biopsies were performed.

Genotype	Number	Histological activity (nos. of patients)	
		Inactive/mild	Moderate/severe
1	20	8 (40.0%)	12 (60.0%)
2	7	3 (42.9%)	4 (57.1%)
3	29	9 (31.0%)	20 (69.0%)
4	54	11 (20.4%)	43 (79.6%)
5	6	1 (16.7%)	5 (83.3%)
Totals	116	32 (27.6%)	84 (72.4%)

6.3 Genotyping of Libyan Haemodialysis Patients

Among the six Libyan haemodialysis patients with HCV infections (Chapter 5, Section 5.2.3), the two females and one male (patients 1, 2 and 6, Table 5.9) were found to have genotype 4. Of the remaining three patients (all male), patient 3 had type 3, patient 4 had type 2, and patient 5 had type 1. There were no apparent correlations between the infecting genotype and serum biochemical liver test results or the other parameters investigated in these patients.

6.4 DISCUSSION

As the hepatitis C virus has not yet been cultured *in vitro* it has not been possible to apply traditional methods for its classification, and progress in this area has had to rely on nucleotide sequence analysis. This has been achieved by a variety of methods, including restriction fragment length polymorphism (RFLP) analysis, type-specific oligonucleotide probing (TSP), and direct sequence analysis. From data obtained using these techniques, a standardized system of HCV nomenclature has recently been agreed upon that reflects the two-tiered sequence of variability (Simmonds et al. 1994a). All known variants of the virus have been classified into a total of six major genotypes (types 1 to 6), of which four (types 1 to 4) each contain a number of more closely related subtypes (e.g. a, b, c, etc.).

In the present study, genotyping was initially performed by RFLP analysis and the data obtained were used for comparing the HCV genotypes in the Libyan subjects with those in patients from other parts of the world. The main drawback of this technique is that there are few (if any) consistent differences in the 5'-noncoding region (5'UTR) sequences between subtypes within a single genotype (e.g. between types 1a and 1b) and it has only very recently become possible to differentiate reliably between different subtypes by RFLP analysis (Davidson et al. 1995). Thus, comparisons could be made only between the major genotypes, without attempting to identify subtypes.

The results clearly showed that genotype 4 predominates in Libya. This is in keeping with previous reports that type 4 is the major genotype in the neighbouring countries of Egypt and the Middle East in general (Simmonds, 1995), which was also confirmed in the present studies of patients from these areas. Of the present patients from the Asian sub-continent, on the other hand, all were found to have type 3. This high prevalence of type 3 in this area has also been described previously (Simmonds, 1995). The highest prevalence of type 3 so far reported outside of Asia has been among intravenous drug users (IVDUs) in Scotland (Simmonds, 1995). Unfortunately, only two Scottish patients were included in the present study and, although both were IVDUs, they both had type 1.

Interestingly, however, all of the patients from elsewhere in Europe who had type 3 were IVDUs. In particular, 11 (91.7%) of the 12 English IVDUs had type 3. Clearly type 3 has

spread to the English IVDU population, but whether this is via IVDUs from Scotland or has been due to the increased travel to Asia (particularly India and Thailand) in recent years is unknown.

Type 5 was previously reported to predominate in Southern Africa (Simmonds, 1995; Ohno et al. 1994), and this has been confirmed in the present study - where all of the five patients from The Gambia, Nigeria, Senegal or South Africa had this genotype. The only other patient with type 5 in the present study was an Afro-Caribbean gentleman who was an immigrant from Jamaica to the U.K. There has been very little direct travel between Africa and the West Indies over the past 150 years and there are very few native (first generation) Africans in the Caribbean area. It is not known how long this gentleman had been in the U.K. or whether he might have inadvertently acquired his type 5 infection through contact with a native African in the U.K., but he had no obvious risk factors for acquisition of HCV. However, the finding that he has this genotype raises the intriguing question of whether type 5 may have reached the Caribbean area during the transportation of African slaves more than 150 years ago.

There was no apparent association between infection with any particular HCV genotype and co-infection with GB virus. The highest frequency of GBV infection was found in patients with HCV genotype 3 (16 of 30 = 53.3%) but 11 of these were IVDUs. By comparison, five (23.8%) of the subjects with type 1 (including two IVDUs) and 12 (17.9%) of the 67 with type 4 were GBV-C RNA seropositive, while none of the seven with type 2 and three of the six with type 5 had GBV infection. Thus, although the frequency in the type 3 subjects was significantly higher ($p < 0.001$) than in those with type 4, it seems likely that this was attributable to intravenous drug use rather than a particular propensity for type 3 to favour co-infection. A surprising finding, however, was that none of the 25 Libyan subjects was GBV-C RNA positive while 25% of the Egyptian patients apparently had GBV infection. The reasons for this large discrepancy in the prevalence of this virus between two neighbouring countries are unclear.

CHAPTER 7

SUB-TYPING OF HEPATITIS C VIRUS GENOTYPES

7.1 INTRODUCTION

7.2 COMPARISON OF GENOTYPES BY DIFFERENT METHODS

7.3 DISCUSSION

7.1 INTRODUCTION

Sub-types of the hepatitis C virus genotypes were determined in all 131 HCV-infected subjects, partly to confirm the results of the RFLP genotyping (Chapter 6) and partly to determine whether there might be important differences between subjects with different sub-types. Intially, sub-typing was performed according to Okamoto et al [1992] but this method does not detect genotypes 3, 4 and 5. The exercise was therefore repeated using type-specific primers (TSP). Details of these two methods are provided in Chapter 3 (Sections 3.2.4.2 and 3.2.4.3) but, briefly, they differ with respect to the sequences of the primers and stringency of the PCR conditions used.

Sub-types were determined from the electropherotype patterns shown diagrammatically in Figs. 7.1 and 7.3 and the examples of electrophoresis gels shown in Figs. 7.2 and 7.4. Where there was more than one pattern of bands, these are defined here as "mixed types". When no bands were obtained (i.e. no amplification of product) results are reported as "undefined".

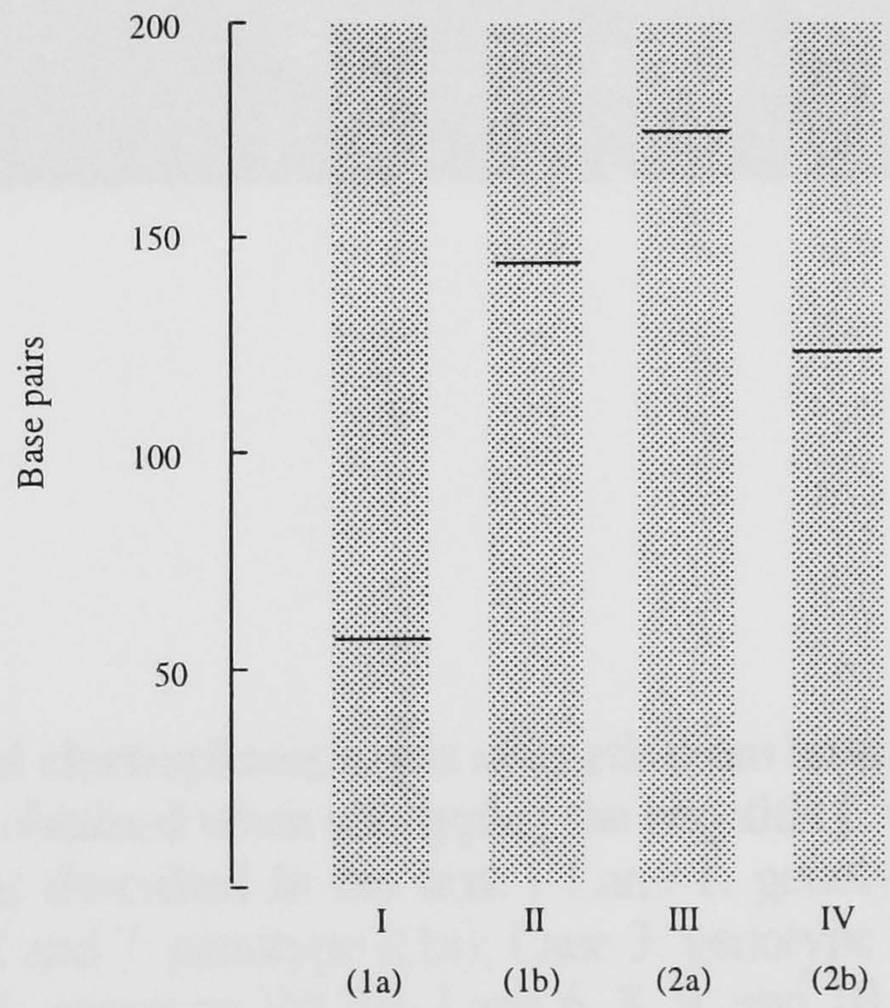


Figure 7.1 **Diagrammatic representation of the electrophoretic patterns produced by hepatitis C virus genotypes I to IV using the method of Okamoto et al as described in the text.** The corresponding types according to the Simmonds classification are shown in brackets.

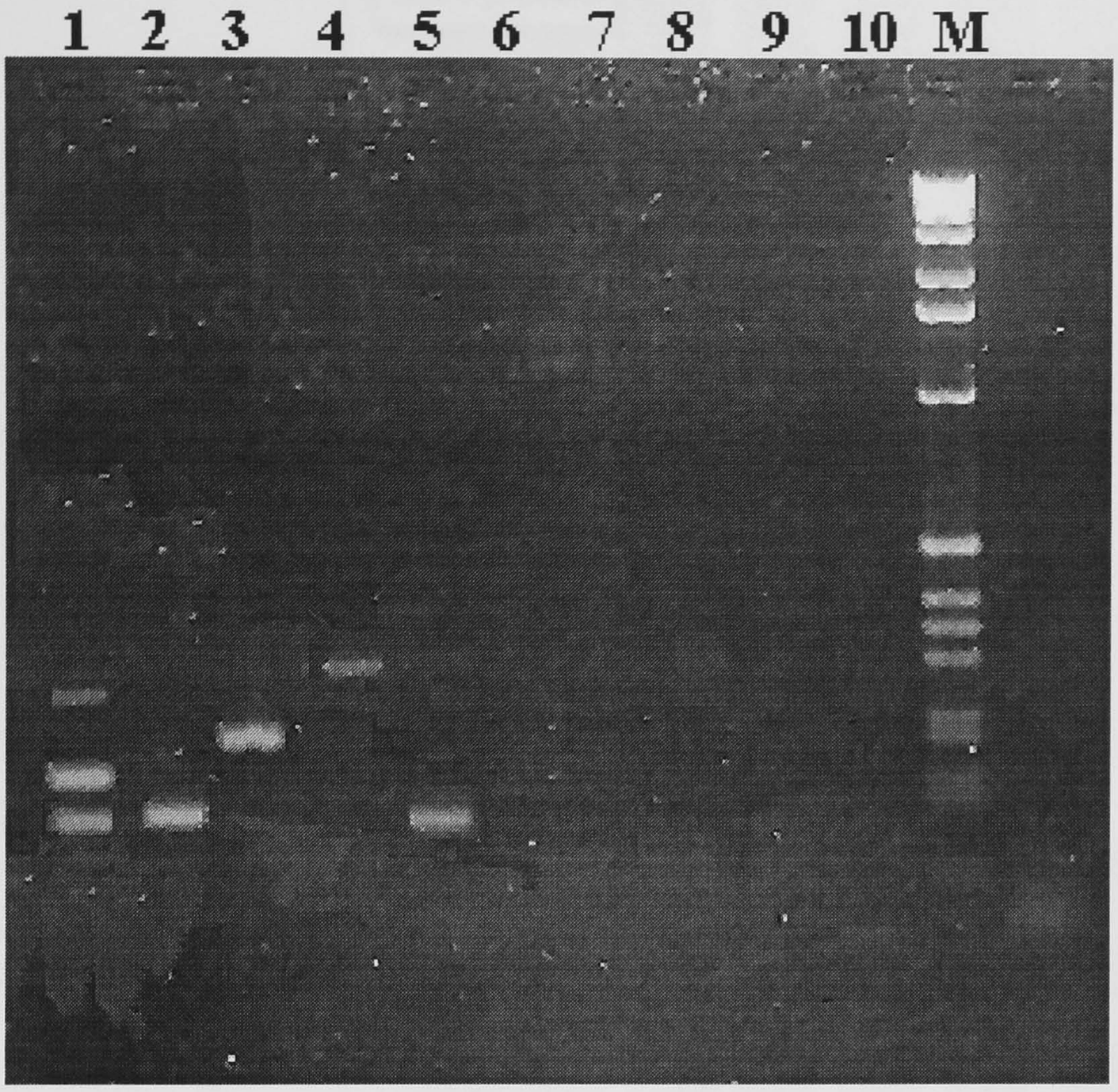


Figure 7.2 **Typical electrophoresis gels after ethidium bromide staining showing patterns of bands obtained when sub-typing the hepatitis C virus by the method of Okamoto et al as described in the text.** [Lane 1: genotype mixed[I(1a)+II(1b)+III(2a)]; Lane 2 and 7: genotype I(1a); Lane 3: genotype II(1b); Lane 4: genotype III(2a); Lane 5: genotype IV(2b); Lane 6, 8, 9, and 10: undefined genotypes.

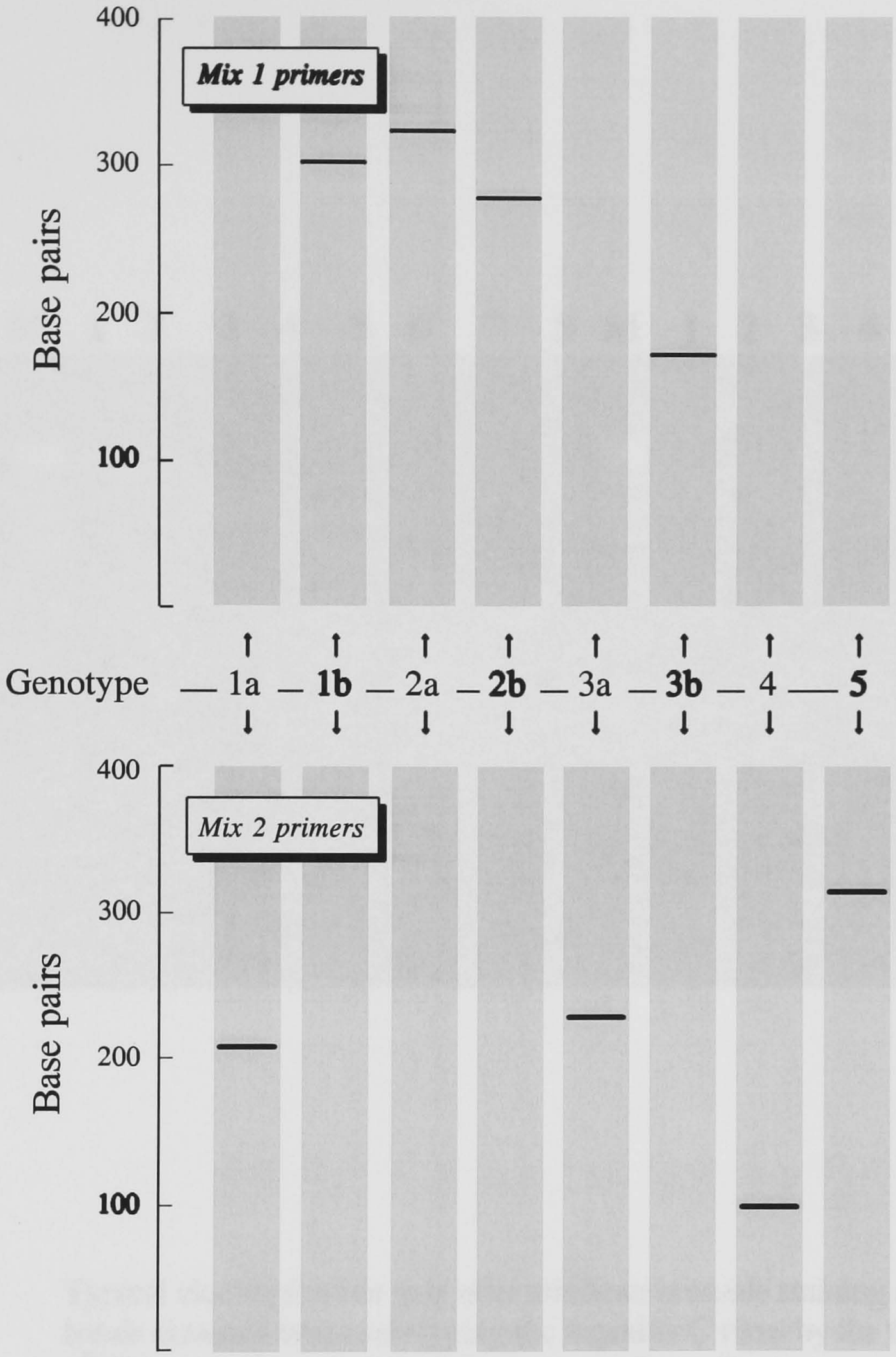


Figure 7.3 **Diagrammatic representation of the electrophoretic patterns produced by different hepatitis C virus sub-types by the type-specific primer (TSP) method as described in the text.**

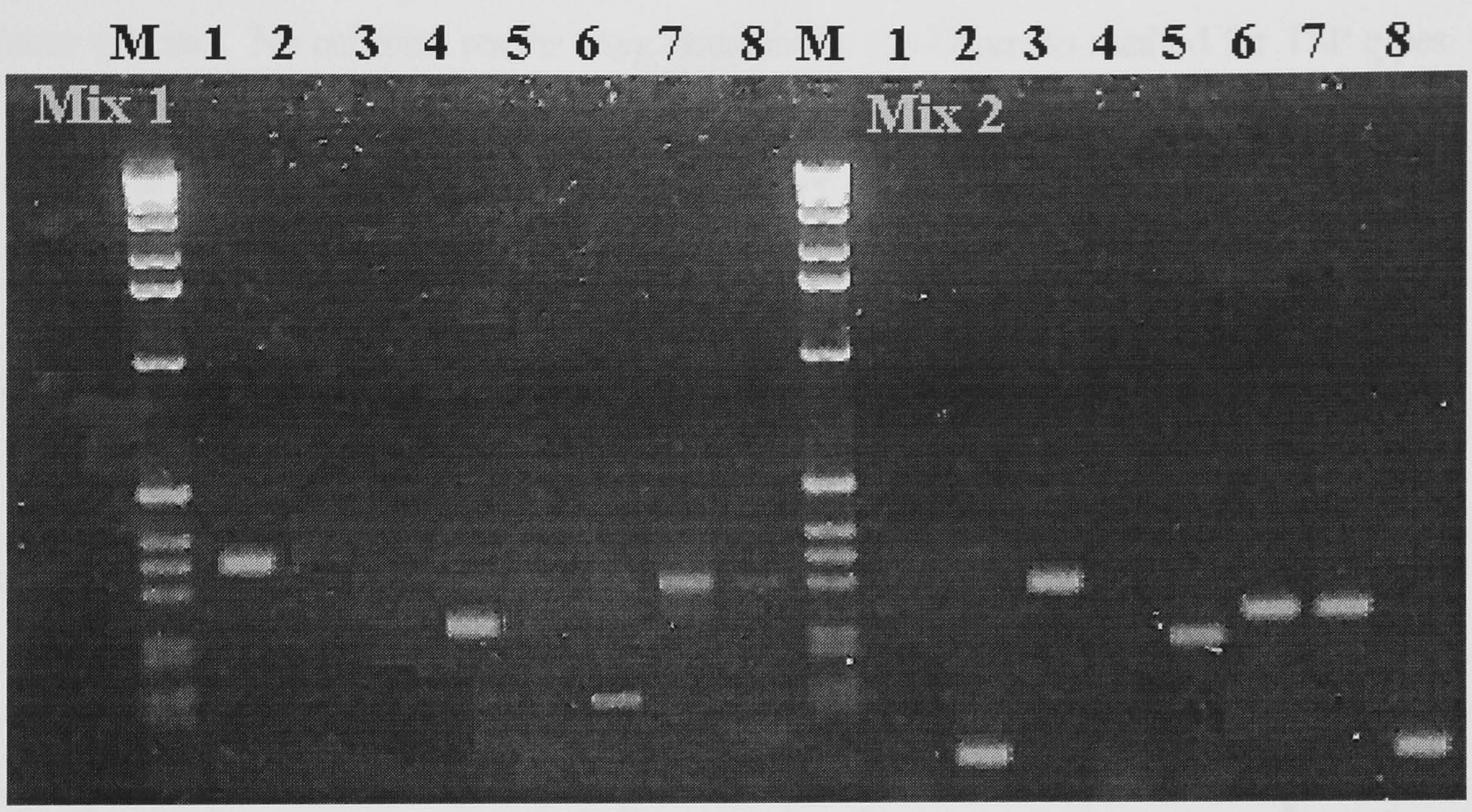


Figure 7.4 **Typical electrophoresis gels after ethidium bromide staining showing patterns of bands obtained when sub-typing the hepatitis C virus by the type-specific primer (TSP) method as described in the text**. [Lane 1: genotype 1b; Lane 2, and 8: genotype 4; Lane 3: genotype 5; Lane 4: genotype 2b; Lane 5: genotype 1a; Lane 6, and 7: genotype 3a.]; M=Marker 1 kb ladder.

7.2 Comparison of Genotypes by Different Methods

For those sub-types where it was possible to make direct comparisons, i.e. types 1a, 1b, 2a and 2b, there was good concordance between the Okamoto and TSP methods. Thus, 24 of 26 subjects with these four sub-types were identified as such by both methods (shaded boxes in Table 7.1). The two anomalies were: one TSP type 2a which was defined as a "mixed" type (1a + 1b) by the Okamoto method, and one TSP type 2b which appeared to be type 2a in the Okamoto system. No coherent results were obtained by the Okamoto method for TSP types 3, 4 and 5. Of the 82 subjects identified by TSP as having these latter three types, one was defined by the Okamoto method as type 1a, three as type 1b and two as type 2a. Forty-five of the remainder gave mixed patterns and 31 were undefinable.

Table 7.1 Comparison of results of hepatitis C virus genotype sub-typing by the type-specific primer (TSP) and Okamoto methods

Sub-typing by Okamoto method	Sub-typing by TSP System										Totals by Okamoto
	1a	1b	2a	2b	3a	3b	4	5	1b+2a+3a	Undefined	
1a	15	-	-	-	-	-	1	-	-	-	16
1b	-	5	-	-	2	-	1	-	-	1	9
2a	-	-	2	1	-	-	2	-	-	1	6
2b	-	-	-	2	-	-	-	-	-	-	2
1a+1b	-	-	1	-	6	11	4	-	-	-	22
1a+2a	-	-	-	-	2	-	1	-	-	1	4
1a+2b	-	-	-	-	2	2	3	-	2	-	9
1b+2a	-	-	-	-	-	1	1	-	-	-	2
2a+2b	-	-	-	-	-	2	5	-	2	-	9
1a+1b+2b	-	-	-	-	1	-	1	-	-	1	6
1b+2a+2b	-	-	-	-	-	-	3	-	-	1	4
Undefined	-	-	-	-	-	2	24	5	5	9	45
Totals by TSP	15	5	3	3	13	18	46	5	9	14	131

In contrast, there was very good agreement between the results of TSP sub-typing and genotyping by RFLP analysis. The main discrepancy occurred in the type 4 patients where there was only 68.7% concordance, with nine RFLP type 4 subjects showing "mixed" (1a + 2a + 3a) electropherotype patterns by TSP and 12 giving patterns that were undefinable. Overall, however, concordant results were obtained in 108 (82%) of the 131 subjects (Table 7.2). In view of these findings, the results obtained with the TSP method were used for analysis of the relationship between sub-types of the virus and the various demographic and clinical features of the subjects.

Table 7.2 Comparison of results of hepatitis C virus genotyping by RFLP analysis and by sub-typing with the type-specific primer (TSP) method

Genotype by RFLP analysis	Sub-typing by TSP System										Totals by RFLP
	1a	1b	2a	2b	3a	3b	4	5	1b+2a+3a	Undefined	
1	15	6	-	-	-	-	-	-	-	-	21
2	-	-	3	3	-	-	-	-	-	1	7
3	-	-	-	-	12	18	-	-	-	-	30
4	-	-	-	-	-	-	46	-	9	12	67
5	-	-	-	-	-	-	-	5	-	1	6
Totals by TSP	15	6	3	3	12	18	46	5	9	14	131

Three of the nine mixed types were Egyptians and the remainder were Libyan cases. Of the 12 RFLP type 4 subjects who were undefined by TSP, seven were from the Middle East and five were Egyptians (Table 7.3). A further patient from the Middle East (Jordan) who was undefinable by TSP had type 2 by RFLP. The remaining undefinable case was the lone patient from South Africa who had type 5 by RFLP. It is noteworthy that all but two of the 23 cases that were either undefinable or defined as "mixed" type by the TSP method had RFLP type 4. These represented 31.3% of the 67 RFLP type 4 cases.

Table 7.3 Geographical distribution of hepatitis C virus genotypes and sub-types

Group	Genotypes and sub-types										Total for Group
	1a	1b	2a	2b	3a	3b	4	5	Mixed	Undefined	
Libya	-	1	-	1	2	-	14	-	6	1	25
Egypt	-	-	-	-	-	2	22	-	3	5	32
Middle East	-	-	-	-	2	-	10	-	-	7	19
U.K.	12	2	1	6	5	-	-	-	-	-	27
Southern Europe	1	2	1	1	-	3	-	-	-	-	8
Sub-Saharan Africa	-	-	-	-	-	-	-	4	-	1	5
Asia	-	-	-	-	2	8	-	-	-	-	10
Carribbean Area	2	1	1	-	-	-	-	1	-	-	5
Totals for type	15	6	3	3	12	18	46	5	9	14	131

There were no striking associations between any one sub-type and presumed modes of infection (Table 7.4). In particular, of the 14 IVDUs with type 3, six had 3a and eight had 3b.

Table 7.4 Analysis of hepatitis C virus genotypes and sub-types with respect to presumed mode of infection

Group	Sub-typing by TSP System										Total for Group
	1a	1b	2a	2b	3a	3b	4	5	Mixed	Undefined	
Blood transfusion	7	2	-	-	3	2	12	-	2	5	33
Intravenous drug use	3	-	-	-	6	8	-	-	-	-	17
Occupational exposure	1	-	1	1	-	2	1	-	-	-	6
Unknown ("Sporadic")	4	4	2	2	3	6	33	5	7	9	75
Totals for type	15	6	3	3	12	18	46	5	9	14	131

Similarly, no significant associations were found between sub-types and severity of liver disease in the 116 patients in whom liver biopsies were performed (Table 7.5). For example, inactive/mild disease was found most frequently in patients with type 2a (2 of 3 = 67.7%) and least frequently in those with type 4 (6 of 37 = 16.2%) but this difference was not statistically significant (p = 0.177). This may have been due to the small numbers but even when groups with larger numbers were compared there were still no significant differences, e.g. type 1a vs. type 4 (6 of 15 with inactive/mild disease = 40.0%, vs. 6 of 37 = 16.2%; p = 0.139).

Table 7.5 **Analysis of hepatitis C virus genotypes and sub-types with respect to histologically assessed disease activity in the 116 subjects in whom liver biopsies were performed**

Histological Activity	**Sub-typing by TSP System**										**Total for Group**
	1a	1b	2a	2b	3a	3b	4	5	Mixed	Undefined	
Inactive/mild	6	2	2	-	4	5	6	-	3	4	32
Moderate/severe	9	3	1	3	7	13	31	5	3	9	84
Totals for type	15	5	3	3	11	18	37	5	6	13	116

7.3 DISCUSSION

The reliability of PCR-based genotyping techniques depends heavily on the specificity of the primers employed and the stringency of the PCR conditions. It is well established that the matching of two or three nucleotides at the 3' end is an important parameter for specific priming. Therefore, the fewer the number of HCV isolates employed in designing a specific primer, the lower will be the specificity of the primers designed. The typing system devised by (Okamoto et al. 1992b) has been considered to be the most appropriate system in Japan and has also been used in several centres around the world. However, it is based on primers corresponding to sequences of HCV isolates derived from individuals infected with genotypes 1a, 1b, 2a, or 2b (Okamoto et al. 1992b). Thus, the Okamoto system is unlikely to identify genotypes from countries where HCV types other than types 1 or 2 are prevalent. Indeed, (Lau et al. 1995) have recently reported that the Okamoto system yields a high number of "mixed" types which they found to be either non-specific assignments of type 1b or types other than 1 or 2. The new TSP system used here is based on nucleotide sequences derived from all of the representative genotypes so far reported and therefore has the capability to identify a much wider range of genotypes. The findings confirm those of (Wan and Mathews, 1994), but on a much larger scale, with 45 (91.8%) of the 49 subjects who were assigned as having "mixed" types by the Okamoto system being found to be neither type 1 or 2 when typed by the TSP system.

Nonetheless, experience has shown that there are always some serum samples where genotype cannot be determined whatever system is used (Davis et al. 1994). This is illustrated in the present study where, of the 45 subjects in whom the genotype could not be defined by the Okamoto system, nine (20%) were also undefinable even by the TSP method. There are several possible reasons for this. Firstly, initial diagnosis of HCV infection relies on seropositivity for anti-HCV antibodies and, in some anti-HCV positive sera, the level of HCV-RNA may be too low for detection. But this is unlikely in the present study, because all of the typed samples had previously been tested positive for HCV-RNA by the nested PCR reaction. Secondly, although the primers for genotyping in the present study were designed from relatively well conserved regions within the core region of each genotype, minor variations between strains within a genotype may still render PCR unsuccessful within the stringency defined for the TSP method. Third, a deletion in the HCV genome might result in amplification of DNA bands of different lengths that do not allow proper genotype assignment. Indeed, during the present study one

patient (who was not part of this study) was encountered who was classified as "indeterminate" by the Okamoto method but who could be assigned as type 1b by TSP typing. Nucleotide sequencing (see Chapter 8) of the core gene from the HCV isolate from this patient revealed a deletion of 117 bp, resulting in a PCR product that was too small for proper genotyping. This might have been due to defective interfering particles but, if this is a new variant, from the available literature it would seem to be uncommon.

Finally, these "hard-to-type" isolates might represent new genotypes that have not yet been identified. To explore this possibility further, PCR products from all of the HCV-RNA positive Libyan patients and all of the other patients classified as having "mixed" or "undefined" types were sequenced. The findings are reported in the next Chapter.

CHAPTER 8

SEQUENCING AND PHYLOGENETIC ANALYSIS OF HEPATITIS C AND GB VIRUS ISOLATES

8.1 INTRODUCTION

As noted in Chapter 7, accurate genotyping and sub-typing of all HCV isolates could not be achieved by RFLP analysis or the TSP method, and a number of HCV isolates from the various groups of subjects studied gave electrophoretic patterns that could not be assigned to a particular genotype and had to be classified as "mixed" or "undefined" (Table 7.3). Direct sequence analysis of the 5'UTR and core regions of HCV genomic material in isolates from the various groups of subjects was therefore undertaken as described in Chapter 3 (Section 3.2.5). This was applied to all of the 25 HCV-RNA positive Libyan control subjects and chronic liver disease patients (Table 7.3) and the six Libyan haemodialysis patients with HCV (Chapter 6, Section 6.3). Of these 31 subjects, two had been assigned as genotype 1 by RFLP ± TSP genotyping analysis, two others as type 2, three as genotype 3, and 17 as genotype 4, while six of the remaining seven were classified as "mixed" and one as "undefined" genotypes. In addition, isolates from all of the 16 non-Libyan patients who had been classified as having either "mixed" or "undefined" genotypes (Table 7.3) were also sequenced.

8.2 SEQUENCE ANALYSIS OF THE HCV 5'UTR REGION

The nucleotide sequences of the PCR products from the 31 Libyan subjects are shown in Table. 8. 2. In the 17 cases who had been assigned to genotype 4 (Table 7.3 and Section 6.3), the sequences were found to be identical and showed 100 % homology with several sequences in GenBank and EMBL(Table 8.1) that have been assigned to genotype 4. Further evaluation of the relationship between these isolates and isolates reported to GenBank was undertaken by constructing a phylogenetic tree (Fig. 8.1), which shows that they all cluster around genotype 4. By comparing the sequences of these isolates with those of previously identified sub-types, it was found that the 17 cases could be further subdivided into five sub-types: 4a, 4b, 4c, 4d, and 4e.

Of the two Libyan subjects who had been initially assigned as having genotype 1, the one who had been assigned to type 1b by TSP typing (Table 7.3) was found to cluster with type 4, and the other showed 97% homology with the published sequence for type 1a. The two who had been assigned to types 2 (one previously classified as type 2b, Table 7.3) and the three who had been assigned to type 3 showed 99% homology with published sequences for types 2a and 3a, respectively. These findings were confirmed by neighbour-joining phylogenetic tree (grow-tree) analysis, in which more than 95% of the bootstrap replicates supported the separation of types 2 and 3 from any of the other sequences.

Finally, the seven Libyan subjects and 14 of the 16 non-Libyan patients who had been initially assigned as having "mixed" or "undefined" types were found to cluster with genotype 4. Of the remaining two non-Libyans, one clustered with type 2 and the other with type 5.

To determine the extent of sequence variability within the 5'UTR of the HCV genome, the above data from the 31 Libyan subjects were combined with that of 20 sequences reported in the literature and a multiple sequence alignment on all 5'UTR sequences was performed. From the resulting consensus sequence, the total nucleotide variation (defined as the total number of nucleotides differing from the consensus sequence) was found to be only 2%. Single and double nucleotide insertions are not uncommon, but sequences of HCV genotypes 1 to 3 were found to be entirely collinear. However, it is noteworthy that, in all of the genotype 4 sequences from the Libyan subjects, there were single base insertions between positions -139 and -138. In addition, there was a nucleotide variation at position -2 in six, but the nucleotide sequence at position + 8 to -65 was invariant among all isolates. In view of the fact that the primary sequence around the AUG initiation codon of a gene is important for initiation of translation(Tsukiyama Kohara et al. 1992), and is a particularly well conserved region, it is also interesting to note that the polyprotein start codon in all of the 31 Libyan isolates occurs at the same location compared with the prototype sequence.

8.3 SEQUENCE ANALYSIS OF THE HCV CORE REGION

To clarify the relationship between different HCV Isolates, the nucleotide sequences of the core region were aligned to maximum homology (Table. 8.3). The numbers of nucleotide substitutions per site at all codon positions were estimated using the 6-parameter method of Saitou and Nei, (1987). Based on these numbers of nucleotide substitutions, a phylogenetic tree was constructed by the neighbour-joining method (Fig. 8.2). The findings completely confirmed those obtained by sequence analysis of the 5'UTR region reported above.

8.4 SEQUENCE ANALYSIS OF THE HCV NS5 REGION

Further evaluation of the phylogenetic relationships between the various genotypes and subtypes was undertaken by sequencing 339 bp fragments of the NS5 region of the HCV genome between nucleotides 7975 and 8313 (Chapter 3, Section 3.2.7) of a limited number of isolates (Table. 8.1). These comprised 14 of the Libyan subjects who had been assigned to genotype 4 and seven non-Libyan patients: two U.K. (both genotype 2), two Pakistan (both genotype 3), and three genotype 5 patients (one each from The Gambia, Nigeria and the Caribbean).

By this analysis, 12 of the 14 Libyan type 4 subjects were found to cluster with genotype 4 (Figs. 8.3 and 8.4), and it was possible to subdivide these into six subtypes (4a to 4f). The remaining two (LIB-30 and LIB-29), both of whom were haemodialysis patients, both clustered with type 1b. Among the non-Libyan patients, the two UK cases clustered with type 2a and the two Pakistanis with type 2b. The three type 5 cases from Africa and the Caribbean were all confirmed as having type 5a.

The additional sequence variation within the NS5 region allows for classification of six types (1 - 6) and 21 distinct subtypes by phylogenetic analysis. However this method is computationally intensive, so simple pair-wise comparisons (which achieves the same results) were made. This showed a wide range of evolutionary distances among the HCV variants tested. Using the DNADIST program from the PHYLIP package (Chapter 3, Section 3.2.7), which directly provides estimates of these molecular evolutionary distances, and based on 1275 pair-wise comparisons, gaps were found between the ranges of evolutionary distances. The gaps ranged from 0.0136 to 1.062 (mean: 0.0599 ± SD 0.0147) within subtypes, 0.1256 to 0.3211(0.2233 ± 0.0149) between different subtypes, and from 0.3662 to 0.8719 (0.6190± SD 0.0124) between different types. The sequence similarity between different types is 56-72%, between different subtypes is 74-86% and within subtypes is more than 88%. Recently Tokita et al. (1994) reported the identification of three other major genotypes (types 7, 8 and 9) on the basis of sequence analysis of the NS5 region of isolates from Vietnamese blood donors but, as can be seen from Fig. 8.4, in the present analysis these were found to cluster with the Hong Kong isolate (HK-2) as new subtypes: 6a to 6e.

8.5 SEQUENCE ANALYSIS OF GB VIRUS

GBV isolates from all of the 40 GBV-C RNA positive sera from the four Libyan haemodialysis patients (Chapter 5, Section 5.4) and the 36 non-Libyan chronic HCV patients, (Chapter 6, Section 6.1.2) were sequenced as described in Chapter 3, Section 3.2.6. Nucleotide sequences of the putative RNA helicase domain were maximally aligned together with HCV types 1 (HCV-1), 2 (HC-J6) and 3 (NZLI). Using the ODEN version 1.1.1 program, the number of amino acid substitutions per site between all possible pairs of these isolates was estimated according to Kimura's formula: $d = -ln(1-p- 0.2P^2)$, where p is the fraction of different amino acids (Gojobori et al. 1990; Kimura, 1983). From these values, a phylogenetic tree was constructed by the unweight pair-group (UPGMA) method with arithmetic mean(Nei, 1975), and the neighbour-joining method Saitou and Nei, (1987). PHYLIP version 3.5c was used to elucidate evolutionary distance, the genetic distances between pairs were estimated using the PROTDIDT program with DAYOFF PAM matrix, and the phylogenetic tree was constructed using the NEIGHBOUR and DRAWTREE programs. The nucleotide sequences from these patients conformed to published sequences for GBV (Table 8.4), confirming the PCR results. As shown in Fig. 8.5, the phylogenetic analysis of these sequences clearly demonstrates the diversion of GBV from HCV.

8.6 DISCUSSION

Being an RNA virus, HCV is capable of rapid evolution. It has been estimated that its mutation rate is of the order of 1.44-1.92 x 10^3 base substitutions per site per year (Okamoto et al. 1992a; Ogata et al. 1991). Consequently, many variants of the virus have been discovered that can be classified into distinct genetic groups. To date, at least four genotyping systems have been proposed. In one system, HCV isolates have been classified into genotypes I, II, III, IV and V based on comparisons of full-length genomic sequences, with intra-genotypic divergence of < 9.2% and inter-genotypic divergence of > 19.9% (Sakamoto et al. 1994). In another scheme, based on phylogenetic analysis of part of the NS5 region, six major types (1, 2, 3, 4, 5 and 6) are recognized, along with a series of subtypes designated a, b, c, etc. (Simmonds et al. 1993a; Stuyver et al. 1993). A third classification system proposes at least 12 genotypes through phylogenetic analysis of a sequence of 192 amino acids encoded by the El gene (Bukh et al. 1993b). The fourth scheme, based on phylogenetic comparisons of the entire El sequence of 576 bp and an NS5b sequence of 1093 bp, identifies at least nine major groups of HCV, which break down into 23 genotypes (Tokita et al. 1994). Whether classification into such large numbers of variants has virological, clinical or epidemiological significance is not yet clear (for further discussion please see Chapter 9).

The data from the present sequence analysis of the 5'UTR and core regions of the HCV genome were entirely confirmatory, and concordance with the genotypic assignations by these methods was found in 17(81%) of the 21 isolates subjected to NS5 analysis. The sequence analyses also confirm the data described in Chapters 6 and 7 indicating that type 4 seems to be the predominant genotype in Libya. Among the 25 HCV-RNA positive Libyan subjects in the control and chronic liver disease groups, 22 (88%) had this genotype. Since few of these subjects had travelled outside their country, it is very likely that the majority of these infections were acquired in Libya. By contrast, only three (50%) of the 6 haemodialysis patients with HCV infection were found to have type 4. Infections with the other genotypes in this group of patients may have been acquired through transfusions with imported blood. Among the 16 non-Libyan patients who had been initially classified as having "mixed" or "undefined & genotypes, 15 were from Egypt or the Middle East (Table 7.3) and all but one of these were found by sequence analysis to have type 4. Thus the present findings are keeping with, and add to, the literature (see Chapter 1, Section 1.4.3) on the prevalence of type 4 in North Africa and the Middle East. Although the majority of the genotype 4 cases appeared to have subtypes 4a or 4b, as reported by (Simmonds et al.

1993b) for isolates from Africa and the Middle East, it is clear that there are several other subtypes of genotype 4.

Genotype 5 has been previously reported in Central and South Africa (Cha et al. 1992; Bukh et al. 1993b; Simmonds et al. 1993a; Ohno et al. 1994) and preliminary data from our laboratories indicate that it is also present in East Africa (Saleh et al. 1994a), but it is believed that this is the first documentation of type 5 in West Africa and the Caribbean. Unlike type 4, relatively little sequence heterogeneity has been found within genotype 5. All isolates that have been analysed so far indicate that there may be only a single subtype (5a) and that infection with this variant is highly restricted to these areas. This apparent sequence homogeneity between type 5 isolates has been confirmed in the present study.

The present study afforded the opportunity to undertake phylogenetic analyses of other published sequences and, particularly, to make comparisons between the new genotypes (7, 8 and 9) proposed by Tokita et al. (1994) (above), by construction of phylogenetic trees based on both the 339 bp and the partial (222 bp) sequences of Simmonds and colleagues (Simmonds et al. 1993a). From Fig. 8.5, it is clear that all of the proposed new genotypes cluster with genotype 6 and probably represent new subtypes (6a to 6d) of genotype 6, rather than distinct new genotypes. It is believed, therefore, that the proposed new genotypes may represent misclassifications.

Finally, from the detailed sequence analyses and phylogenetic trees recently reported for the helicase and RNA-dependent RNA polymerase regions of GBVs A and B by Muerhoff et al. (1995), and for GBV-C by Simons et al. (1995b), the predicted amino acid sequence homologies between HCV and GBV range between 20% and 30%. It is thought that viral proteins containing NTP-binding motifs may be subunits of RNA helicases involved in the unwinding of double-stranded replication forms during RNA replication and in recombination between RNA genomes (Hodgman, 1988; Gorbalenya and Koonin, 1989; Wassarman and Steitz, 1991). It is interesting to speculate that these two viruses may have originated through a common ancestor.

Table 8.1 Designation and accession Number of HCV sequences used in the study

Abbreviation	Accession number	Abbreviation	Accession number
HCV1	D00831	VN085	D17471
GM1	M61720	VN235	D14202
GM2	M61721	VN405	D17472
HCV-J	D00574	VN506	D17473
HC-J6	D00944	VN541	D17474
HCV-BK	M58335	VN569	D17476
LIB-1	D31726	VN571	D17477
Paki-1	D16720	VN606	D17478
Paki-2	D16721	VN655	D17479
Q19	D16811	VN689	D17480
Q14	D16813	VN693	D17481
SA-K3	D16789	VN710	D17482
SA-K14	D16792	VN711	D17483
E-b1	D10123	VN746	D17484
T3	L16648	VN753	D17485
Sa1	L16642	DK13	M84832
Sa7	L16645	EG-13	L08140
HK-10	L16659	Z4	M84848
P8	M84856	Z6	M84862
HK2	L16634	VN004	D17470
Z8	M84829	SA156	L23471
4TY4	L23447	2TY4	L23446
AGR6	L23457	AGR8	L23458
NZL1	D14305	USAGC54	L23453

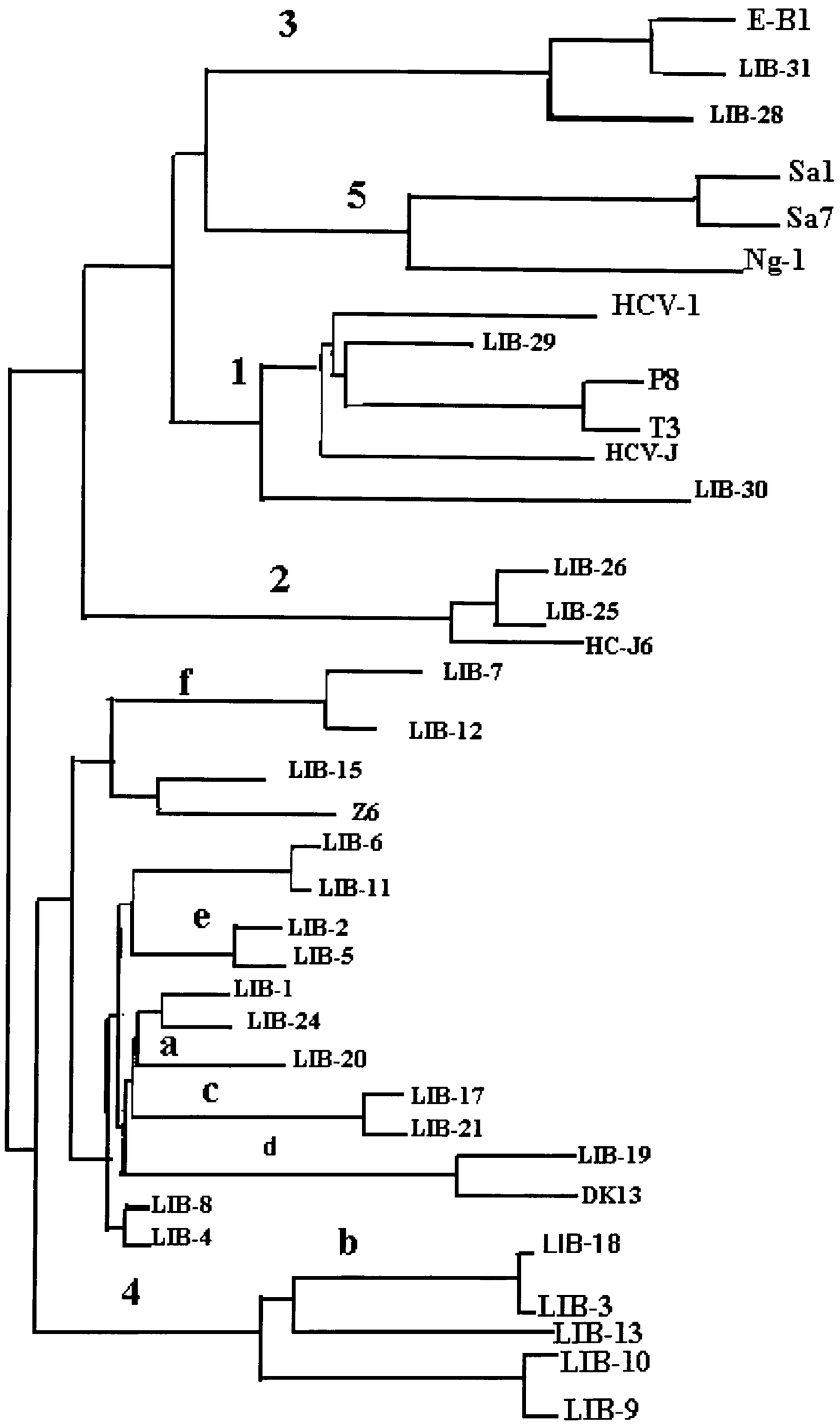


Figure 8.1 **Growtree analysis of 5' UTR sequences of HCV from Libyan** (LIB-n) compared with reported sequences of other isolates (E-B1, Sa-1, Sa-7, HCV-1, P8, T3, Hc-J6, Z6, Dk13), Ng-1, Jam-1, Gam-2, Sa-In this isoltes from this study.

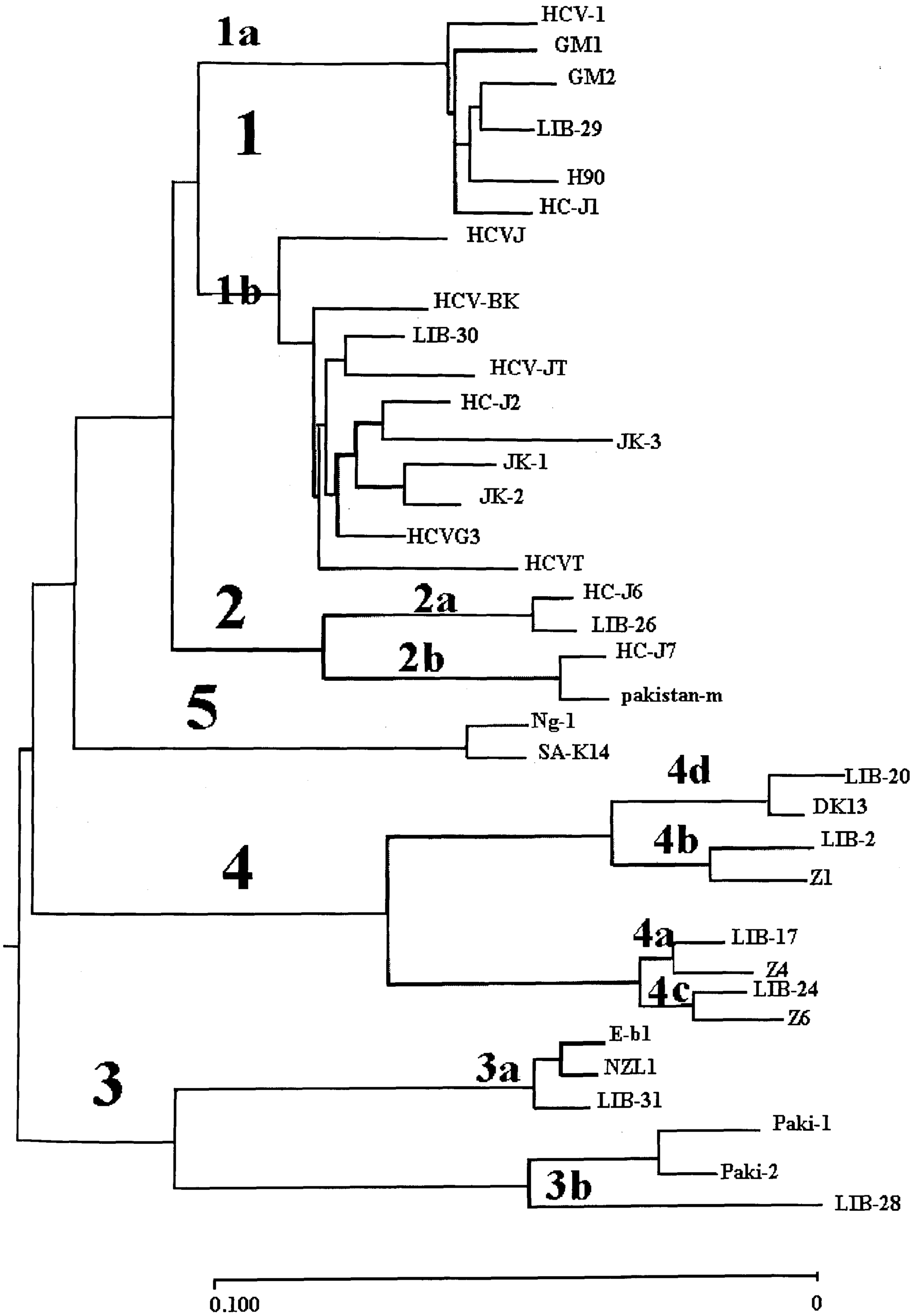


Figure 8.2 Phylogenetic tree was constructed by the neighbour-joining method from the core region

		-244 ▾	-234 ▾	-224 ▾	-215 ▾	-184 ▾	-174 ▾	-169 ▾	-154 ▾	-144 ▾	-137 ▾	-127 ▾	-115 ▾	-100 ▾	-90 ▾	-80 ▾	-69 ▾
HCV-1	1a	TGAGTGTCGT	GCAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATTG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
HCV-J	1b	TGAGTGTTGT	GCAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATTG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCGAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-29	1a	TGAGTGTCGT	GCAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATTG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-30	1b	TGAGTGTCGT	GCAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATTG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCGAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
HC-J6	2a	TGAGTGTCGT	ACAGCCTCCA	GGCCCCCCCC		CGGTGAGTAC	ACCGGAATTG	CCGGGAAGAC	TGGGTCCTTT	C--TTGGAT-	AAACCCACTC	TATGCCCGGT	CAT	GCAAGACTGC	TAGCCGAGTA	GCGTTGGGTT	GC
HC-J8	2b	TGAGTGTCGT	ACAGCCTCCA	GGCCCCCCCC		CGGTGAGTAC	ACCGGAATTA	CCGGAAAGAC	TGGGTCCTTT	C--TTGGAT-	AAACCCACTC	TATGCCCGGT	CAT	GCGAGACTGC	TAGCCGAGTA	GCGTTGGGTT	GC
LIB-25	2b	TGAGTGTCGT	ACAGCCTCCA	GGCCCCCCCC		CGGTGAGTAC	ACCGGAATTA	CCGGGAAGAC	TGGGTCCTTT	C--TTGGAT-	AAACCCACTC	TATGCCCGGT	CAT	GCGAGACTGC	TAGCCGAGTA	GCGTTGGGTT	GC
LIB-26	2a	TGAGTGTCGT	ACAGCCTCCA	GGCCCCCCCC		CGGTGAGTAC	ACCGGAATTG	CCGGAAAGAC	TGGGTCCTTT	C--TTGGAT-	AAACCCACTC	TATGTCCGGT	CAT	GCAAGACTGC	TAGCCGAGTA	GCGTTGGGTT	GC
E-B1	3a	CGAGTGTCGT	GCAGCCTCCA	GGACTCCCCC		CGGTGAGTAC	ACCGGAATCG	CTGGGGTGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATACCCAGA	AAT	GCGAGATCAC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-31		CGAGTGTCGT	GCAGCCTCCA	GGACTCCCCC		CGGTGAGTAC	ACCGGAATCG	CTGGGGTGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATACCCAGA	AAT	GCGAGATCAC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-27		CGAGTGTCGT	GCAGCCTCCA	GGACTCCCCC		CGGTGAGTAC	ACCGGAATCG	CTGGGGTGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATACCCAGA	AAT	GCGAGATCAC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-28		CGAGTGTCGT	GCAGCCTCCA	GGACTCCCCC		CGGTGAGTAC	ACCGGAATCG	CTGGGGTGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATACCCAGA	AAT	GCGAGATCAC	TAGCCGAGTA	GTGTTGGGTC	GC
EG-14	4a	TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
EG-16		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
IQ-48		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
EG-21		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
EG-96		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
EG-28		TGAGTGTTCT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-1		TGAGTGTTCT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-2		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-3		TGAGTGTTCT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-4		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-5		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-6		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-7		TGAGTGTTGT	CCAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-8		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-9		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-10		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-11		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-12		TGAGTGTTCT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-13		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-14		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-15		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-16		TGAGTGTTCT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-17		TGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGCAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-18		TGAGTGTTGT	ACAGCCTCCA	GGACCCTCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-19		TGAGTGTTCT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGCAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-20		CGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGCAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-21		CGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-22		CGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGCAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-23		CGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGCAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC
LIB-24		CGAGTGTTGT	ACAGCCTCCA	GGACCCCCCC		CGGTGAGTAC	ACCGGAATCG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-	CAACCCGCTC	AATGCCTGGA	GAT	GCAAGACTGC	TAGCCGAGTA	GTGTTGGGTC	GC

Table 8.2 nucleotide of the sequences in 5'UTR of HCV for Libyan patients. [Bold indicates Substitutions, - indicate gap]

Table 8.3 **The nucleotide sequences of the core region aligned with maximum homology**

	29	39	49	59	69	79	89	99	109	119	129	139
HCV-1	AAAACAAACG	TAACACACCAAC	CGTCGCCCAC	AGGACGTCAA	GTTCCCGGGT	GGCGGTCAGA	TCGTTGGTGG	AGTTTACTTG	TTGCCGCGCA	GGGGCCCTAG	ATTGGGTGTG	CGCGCGACGA
LIB-29	--G-------	----------	----------	------G---	----------	----------	----------	----------	----------	----------	----------	----------
HCV-BK	--C-------	----------	--C-------	-------T--	---------C	--T-------	----------	-------C--	----------	-------C--	G---------	--------T-
LIB-29	--C-------	----------	--C-------	-------T--	---------C	--A-------	----------	-------C--	----------	-------C--	G---------	--------T-
HC-J6	--C-----A-	A---------	-TG-------	-A-----T--	---T-----C	-----C----	-------C--	---A------	----------	-------C--	G---------	--------A-
LIB-26	--C-----A-	A---------	-TG-------	-A-----T--	---T-----C	-----C----	-------C--	---A------	----------	-------C--	G---------	--------A-
HC-J7	--C-----A-	A---------	--C-------	-------T--	----------	----------	-------C--	----------	C---------	-------C--	G---------	--------A-
pakis-m	--C-----A-	A---------	--C-------	-------T--	----------	----------	-------C--	----------	C---------	-------C--	G---------	--------A-
EG-13	--C-------	----------	--C-----CA	T------T--	----------	--T--C----	-------C--	----------	----------	--------C-	T---------	--------TC
LIB-2	----------	----------	--C-----CA	T------T--	----------	--T---C---	-------C--	---AA----T	-------T--	-------T--	---------C	----G-----
LIB-17	----------	----------	--C-----CA	T------T--	----------	--T---C---	-------C--	---------T	-------T--	G--------T	--T-------	-------G--
LIB-20	----------	----------	--C-----CA	C------T--	---T------	--T---C---	-------C--	CA-------T	-------T--	T---------	--------C-	----A--G--
LIB-24	----------	----------	--C-----CA	T------T--	-A--------	--T---C---	-------C--	CA-------T	-------T--	G--------T	--T-------	-------G--
E-B1	--C-----A-	A-------T-	----------	----------	----A-----	----------	---A---G--	----------	-------AC-	----------	T--------C	----------
LIB-31	--C-----A-	A-------T-	----------	----------	----A-----	----------	---A---G--	----------	-------AC-	----------	T--------C	----------
LIB-28	--C-----A-	A-------T-	----T-----	A--------C	----A-----	----------	---G--G---	---------T	-------AC-	-------T--	T--------C	----------

	149	159	169	179	189	199	209	219	229	239	249	259
HCV-1	GAAAGACTTC	CGAGCCGTCG	CAACCTCGAG	GTAGACGTCA	GCCTATCCCC	AAAGCTCGTC	GGCCCGAGGG	CAGGACCTGG	GCTCAGCCCG	GGTACCCTTG	GCCCCTCTAT	GGCAATGAGG
LIB-29	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
HCV-BK	-G--------	----------	--------T-	-A--G--A--	A---------	--------C-	----------	T---------	----------	----------	----------	-----C----
LIB-29	-G--------	----------	--------T-	-A--G--A--	A---------	--------C-	----------	T---------	----------	----------	----------	-----C----
HC-J6	-G--------	G--------C	--G--A--T-	-A--G--C--	---C-----T	----A---G-	-CT--ACT--	--AAT-----	-GAA-A--A-	-A-----C--	------A--C	--G--C----
LIB-26	-G--------	G--------C	--G--A--T-	-A--G--C--	---C-----T	----A---G-	-CT--ACT--	--AAT-----	-GAA-A--A-	-A-----C--	------A--C	--G--C----
HC-J7	-G--------	T-----A--C	--G--G--T-	-AC----C--	---C-----G	----A---G-	-CT--ACT--	--A-T-----	-GAA----A-	-A--T-----	------G--C	--A--C----
pakis-m	-G--------	T-----A--C	--G--G--T-	-AC----C--	---C-----G	----A---G-	-CT--ACT--	--A-T-----	-GAA----A-	-A--T-----	------G--C	--A--C----
EG-13	-G--------	G---------	--------T-	-G-----C--	A---------	--G--G----	-AT-------	A---T-----	--A--A--A-	-A--T--A--	---T--T--C	--T-------
LIB-2	-G--------	G---------	--------T-	-G-----C--	A---------	--G--G----	-AT-------	A---T-----	--A--A--A-	-A--T--A--	---T--T--C	--A--A----
LIB-17	----------	G---------	--------T-	-G-----C--	A---------	--G--G----	-AT-------	A---T-----	--A--A--A-	-A--T--A--	----------	--A--A----
LIB-20	----------	G---------	--------T-	-G--------	----------	--G--G----	-AT-------	A---T-----	--A-----A-	-A-TT--A--	---T--T--C	--A--A----
LIB-24	-G--------	G---------	--------T-	-G--------	----------	--G--G----	-AT-------	A---T-----	--A-----A-	-A-TT--A--	------T--C	--A--A----
E-B1	-T--A-----	T--A-----A	--G-----C-	-AC----A--	----------	--AG------	---T------	----------	-------G--	----------	--T--C----	----------
LIB-31	-T--A-----	A--A-----A	--G-----C-	-AC----A--	----------	--AG------	---T------	----------	-------G--	----------	--T--C----	----------
LIB-28	-T--A-----	A--A-----A	--G-----C-	-AC----A--	----------	--AG------	---T------	----------	-------G--	----------	--T--C----	----------

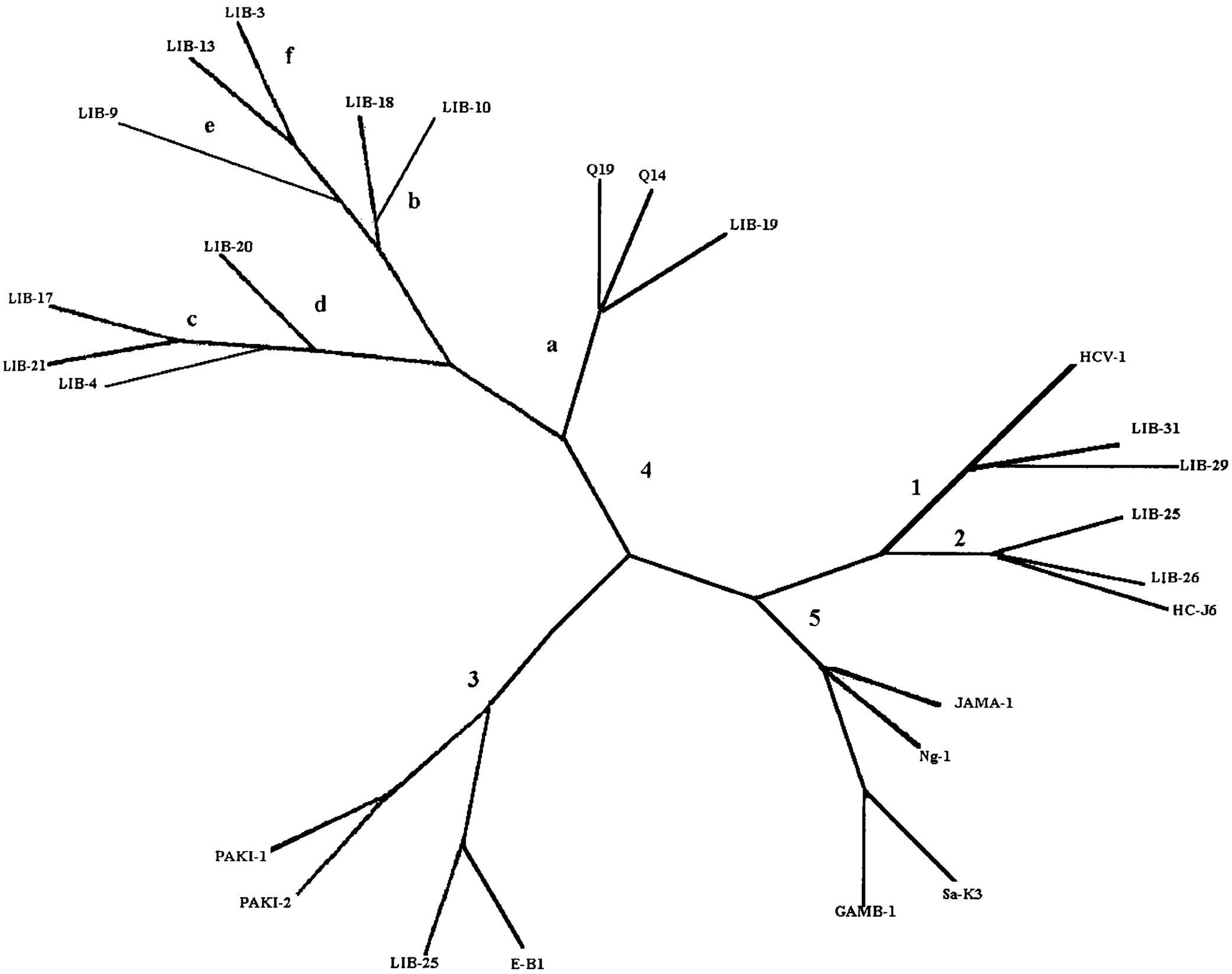


Figure 8.3 **Phylogenetic tree of the nucleotide sequences in HCV NS5-region for the Libyan and non-Libyan patients**

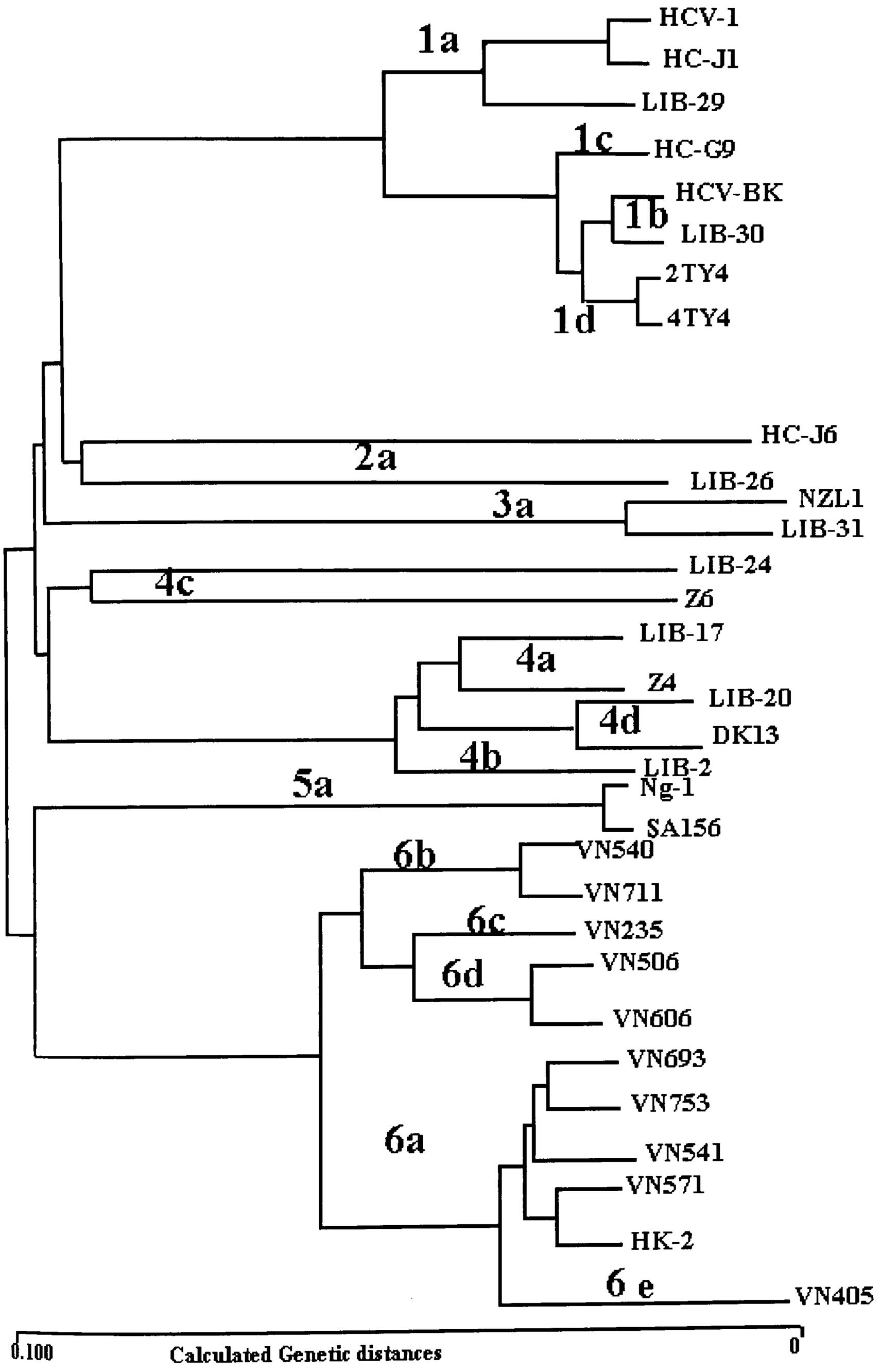


Figure 8.4 Phylogenetic analysis for the comparison with Tokita et al Genotypes.

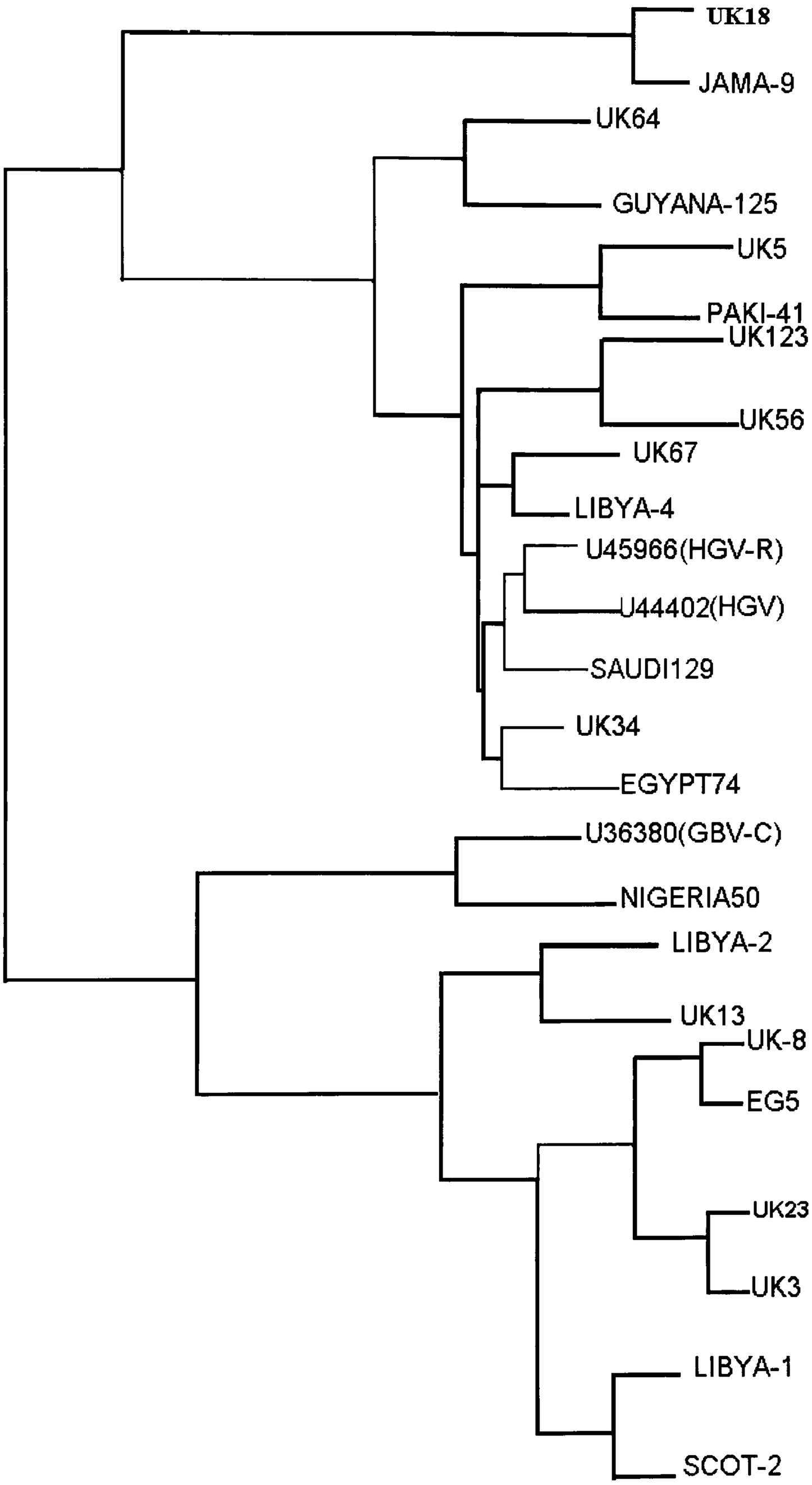


Figure 8.5 Phylogenetic tree of GBV-C/HGV.

Table 8.4 Nucleotide sequences for GBV-C/HGV

HGV(U44402)	CGAGCGGATG	CGAACCGGAA	GGCACCTCGT	GTTCTGCCAT	TCTAAGGCTG	AGTGCGAGCG	CCTTGCTGGC	CAGTTCTCCG	CTAGGGGGGT	CAATGCCATT	GCCTATTATA	GGGGTAAA
UK18	----------	----------	----T-----	T--------C	--CA------	----------	----------	-----T----	------A---	T--------T	----------	----T-----
UK3	G---------	--------T-	----------	---------T	--A-----T-	----------	---G--G---	---------T	-------T--	-------T--	---------C	----G-----
UK34	T---A-----	----------	----C-----	G--C-----T	--------G-	----------	-----T----	--------A-	-C-------A	G--------T	--T-------	-------G--
UK64	T---------	--G-----T-	----------	A---------	--A-----G-	----------	T-----C--T	-----T---T	-C--------	T---------	--------C-	----A--G--
UK67	T---------	--G--T--G-	----T-----	A--------C	--A-------	----------	---G--A---	--A-----T-	-C-------A	G--------T	--T-------	-------G--
UK5	G---------	--------T-	----------	A---------	--A-----G-	----------	T-----C--T	-----T---T	-C-------A	T---------	--------C-	-------G--
UK123	T---------	--G-----G-	----------	A---------	----------	----------	---G------	--A-------	-G--------	G--C-----C	---T-----C	----------
UK56	T---------	--G-----G-	----------	A---------	----------	----------	---G------	--A-------	-G--------	G--C-----C	---T-----C	----------
UK23	G---------	--------T-	----------	---------T	--A-----T-	----------	---G--G---	---------T	-------T--	----------	---------C	----G-----
UK8	G-------C-	--------T-	----------	---------T	--A-----T-	----------	---G--G---	---------T	-------T--	T---------	---------C	----G-----
UK13	G-------C-	--------T-	C---------	---------T	--A-----T-	----------	---G--G---	---------T	-------T--	T---------	---------C	----G-----
SCOT-2	G---------	--------T-	----------	---------T	--A-----T-	----------	---G--G---	---------T	-------T--	T---------	---------C	----G-----
LIBYA-1	G---------	--------T-	----------	---------T	--A-----T-	----------	---G--G---	---------T	-------T--	T---------	---------C	----G-----
LIBYA-2	G-------C-	--------T-	C---------	---------T	--A-----T-	----------	-----T----	--A------T	-------T--	T---------	---------C	----G-----
LIBYA-4	T---------	--G--T--G-	----T-----	A--------C	--A-----G-	----------	---G--G---	---------T	-C-------A	-------T--	--T------C	----T-----
EGYPT-74	T---A-----	----------	----C-----	G--C-----T	----------	----------	---G--G---	---------T	-------T--	-------T--	----------	-------G--
EG5	G-------C-	--------T-	----------	---------T	--A-------	----------	---G------	-----T---T	-G--------	G---------	--T-------	----------
SAUDI129	----------	----------	----------	----------	--CA------	----------	---G------	--A------T	-------T--	G--C-----C	----------	----G-----
NIGERIA50	---A------	----------	----------	----------	---A----T-	----------	T-----C--T	--------A-	----------	----------	--T------C	----A--G--
PAKI-41	T---------	--------T-	----------	A---------	----------	----------	T-----C--T	---------T	-G--------	G--C-----C	----------	----------
GUYANA-125	T---------	--------T-	----------	A--------C	----------	----------	----------	----------	----------	----------	----------	----------
JAMA-9	----------	----------	----------	T--------C	----------	----------	----------	----------	----------	----------	----------	----------

CHAPTER 9

FINAL DISCUSSION

9.1 VIRAL HEPATITIS IN LIBYA

9.2 TRANSMISSION OF THE HEPATITIS C VIRUS

9.3 GENOMIC VARIATION OF THE HEPATITIS C VIRUS

9.4 CLINICAL SIGNIFICANCE OF GENOMIC VARIATION OF THE HEPATITIS C VIRUS

9.5 FUTURE STUDIES

9.1 VIRAL HEPATITIS IN LIBYA

It seems clear from this study that the background prevalence of two of the viruses capable of causing chronic hepatitis, i.e. HBV and HCV, in the Libyan population must be high. The socioeconomic implications of this are significant. Unfortunately, economic strictures to date have dictated that screening for hepatitis viral infections is limited to testing for HBsAg and only by a relatively insensitive test (Wellcotest). In the present study, some 13.2% of the "healthy" blood donors studied had evidence of current infection with either HBV or HCV. Therefore, until more rigorous and comprehensive screening procedures are introduced, HBV and HCV infections will continue to be spread in Libya through blood transfusions. Additionally, particularly against this economic background, accurate diagnosis in infected individuals would seem essential. Interferon therapy, although so far somewhat disappointing in chronic HCV infection, is effective in many cases of chronic hepatitis B (Di Bisceglie et al. 1990), and targeting of this expensive treatment to those individuals most likely to benefit seems sensible. In this regard, it is worth noting that 25 % of the Libyan patients who were considered to be cases of non-A, non-B hepatitis were in fact HBsAg seropositive on re-testing (Chapter 5, Section 5.3.2).

Interestingly, the two other viruses known to be capable of causing chronic hepatitis, HDV and GBV, appear to be very rare in Libya. As noted in Chapters 1 and 5, intravenous drug use is a major risk factor for acquisition of these viruses, and their relative absence in Libya might be related to the low incidence of intravenous drug use in that country. It is unlikely, however, that this alone can account for the low frequency of detection in this population of GB virus, which is reportedly quite widespread in Egypt and elsewhere in Africa (Zuckerman, 1995).

The finding of a high frequency of exposure to HBV in this population is not surprising, given that this virus is endemic in North Africa. However, the prevalence and nature of HCV infection in Libya has not been investigated previously and the present study has therefore concentrated on hepatitis C virus infection.

9.2 TRANSMISSION OF THE HEPATITIS C VIRUS

All of the Libyan subjects with HCV seem to have been cases of "sporadic" infection (Chapter 5). How such infections are acquired remains a mystery. There is no evidence that the virus is capable of infection other than by the parenteral route. Inapparent transmission through close contact between individuals with open sores or abrasions, particularly in children during play, is one possibility. However, none of the 49 subjects under the age of 20 years in the present study had evidence of exposure to HCV. As noted in Chapter 1 (Section 1.4.2), data on sexual and maternal/foetal transmission are conflicting but, overall, transmission via these routes appears to be low. Nonetheless, there does seem to be an increased incidence of exposure to the virus in households with an infected individual. In contrast to other flaviviruses (such as those causing yellow fever and dengue fever) that are spread by mosquitoes or sand flies, few epidemics of hepatitis C have been reported. If transmission involves an arthropod vector, therefore, this is unlikely to be a flying insect. The possibility that HCV infection might be spread by a crawling insect such as a bedbug or house mite needs to be investigated.

One possible explanation for the present findings relates to the practice of men attending barber shops each day to be shaved, which is widespread (almost ritualistic) in Libya. Indeed, in a recent letter to Lancet, (Tumminelli et al. 1995) have reported that 38% of Sicilian barbers who shaved themselves regularly with the same non-disposable and unsterilised blades used on customers had circulating anti-HCV antibodies, compared with none of the 50 age- and sex-matched control subjects studied. This would not, however, explain the high frequency of HCV infection among the female Libyan subjects.

9.3 GENOMIC VARIATION OF THE HEPATITIS C VIRUS

Sequence variation within the HCV genome undoubtedly affects screening for infection by detection of anti-HCV antibodies, because it leads to variability in the protein products of the virus and therefore in the host immune response and the specificity of the antibodies produced. The C100-3 protein on which the first generation anti-HCV test was based was derived from HCV type 1 and identified about 90% of individuals infected with this genotype, but only one third of those with types 2 or 3(McOmish et al. 1993). Later generation assays incorporating peptides from the core region (c22) are much less prone to such false-negativity, presumably reflecting the greater degree of conservation in this region of the genome (Simmonds et al. 1994b). Certainly, in the present study this seems to have been the case, for the UBI assay used detected anti-HCV antibodies in all but three subjects with

circulating HCV-RNA regardless of the genotype with which they were infected.

The present finding that genotype 4 predominates in Libya and that, by the combination of TSP typing, sequencing and phylogenetic analysis, up to six distinct subtypes may exist in that country, is in keeping with the reported distribution of this genotype in this geographical area. However, Simmonds (Simmonds, 1994; Simmonds, 1995) emphasises the extreme difficulties in assigning geographical distributions to the various genotypes and subtypes at the present time. On the one hand, increased tourist travel, general mobility of populations, and interchange of blood products for therapeutic purposes between countries during the past three decades has meant that genotypes that may have been restricted to one area are now found in quite disparate countries. For example, 40% of blood donors in Scotland are reportedly (Simmonds, 1994) infected with type 3 possibly via intravenous drug users following the "hippie" trail through South-East Asia. On the other hand there is considerable heterogeneity between subtypes of certain genotypes. To quote Simmonds, (1995): "although genotypes in the Middle East and Central Africa can be assigned as genotype 4 on the basis of sequence comparisons in the core, El and NS-5 regions, it is apparent that this genotype actually comprises a bewildering number of subtypes..... type 4a is the most frequently found genotype in Egypt and elsewhere in the Middle East, [but] we have detected at least three other subtypes of type 4 in this region alone [and] El sequences from six infected individuals in Zaire yielded four subtypes, none of which correspond to those in the Middle East nor to many of those found in Gabon". These problems present difficulties for phylogenetic analysis to study the evolution of the virus.

9.4 CLINICAL SIGNIFICANCE OF GENOMIC VARIATION OF THE HEPATITIS C VIRUS

Although there is increasing evidence that the clinical profile, severity of liver disease, and response to anti-viral therapy may depend on the particular genotype or subtype with which the patient is infected (Mita et al. 1994; Dusheiko et al. 1994; Mahaney et al. 1994; Davidson et al. 1995), the clinical utility of determining the genotypes and subtypes of HCV in infected individuals is currently the subject of much debate. The available data are somewhat conflicting, and it is often difficult to compare results of the different studies because of major differences in their design and technical execution. One early report suggested that there was no significant difference in severity of liver disease between individuals infected with genotype 1 or type 2 (Takada et al. 1992). A slightly later study indicated that patients with type 3 had a higher rate of liver function abnormalities than those with either type 1 or type 2 (McOmish et al. 1993), while a very recent study from Italy found that asymptomatic carriers of genotype 1 had abnormal biochemical liver tests significantly more frequently than those with type 2,

although the incidence of histologically proven chronic hepatitis did not differ between these two groups (Prati et al. 1996). Several studies in Japan have suggested that infections with genotype 1, and particular subtype 1b, are less susceptible to treatment with interferon (Pozzato et al. 1991; Takada et al. 1992; Kanai et al. 1992; Yoshioka et al. 1992).

This tendency for type 1 patients to have more severe disease and a poorer response to anti-viral therapy was confirmed in a later study of 80 patients from different countries who were variously infected with types 1 to 4, but it was noted that severe disease could be found in association with all four types (Dusheiko et al. 1994). A study in the USA found that patients infected with type 2 had histologically more severe disease, but lower levels of circulating HCV-RNA and a better response to interferon, than those with types 1 or 3 (Mahaney et al. 1994). On the other hand, a more recent study from France (Martinot Peignoux et al. 1995), which confirmed the tendency for patients with type lb and/or high levels of viraemia to have a poor response to interferon, found that older age, infection via blood transfusion (possibly reflecting a higher viral load in the infecting inoculum), and longer duration of infection, were also significantly associated with a poor response. Another recent study, in French and Italian patients (Nousbaum et al. 1995), also confirmed that type lb, older age and longer duration of disease are associated with a significantly poorer response to treatment, but found no difference in levels of viraemia between patients with different genotypes. That study also suggested that the prevalence of type lb in French and Italian patients seemed to be decreasing, being found less frequently in younger patients (Nousbaum et al. 1995). This has implications for the study of the evolution of the virus. However, it is difficult to make such extrapolations from studies conducted at only one time point, which give only a cross-sectional "snapshot" of a particular population. An alternative explanation for the apparent decrease in type lb, suggested by Sallie (Sallie, 1995), is that the infecting genotype is related to the mode of acquisition which, in turn, may be age-related. In support of this, he points to the study of Mahaney et al. (1994) which showed no difference in frequency of different genotypes or subtypes between infected intravenous drug users but a seven-fold higher frequency of type lb among patients who had acquired their infections via blood transfusion.

Overall, it is probably true to say that there is not yet sufficient evidence to draw definitive conclusions about the influence of genotype on severity of disease and outcome. However, the present findings that there were no correlations between histological severity of disease and the infecting genotypes would suggest that the influence of the different genotypes on disease severity may be quite marginal and that other factors may be more important in determining outcome. What these factors may

be is currently unknown. The suggestion that the mode of acquisition of the infection may be important (Gordon et al. 1993) is not borne out by the present finding that there was no correlation between disease severity and the presumed route of infection - which is in agreement with a very recent report by Chiaramonte et al. (1996).

9.5 FUTURE STUDIES

Undoubtedly the present findings point to the need for a full epidemiological survey of, particularly, HCV and GBV infection in Libya. Prevention of transmission via blood transfusions, and accurate diagnosis in patients with suspected viral hepatitis, through proper screening would seem to be of paramount importance - although whether this will be possible in the present economic climate in that country is uncertain. Ultimately, the aim must be to introduce a mass vaccination program but the variability of the HCV genome is presenting problems for the development of effective vaccines and not enough is yet known about GBV to say whether it might be possible to produce a vaccine against this virus. Thus, the realisation of this aim would seem to be still a long way off. Nonetheless, attempts to reduce "sporadic" transmission at relatively little cost might be made if the mode of these infections can be identified. In this context, it would be useful to attempt to investigate whether there is cross-infection in barber shops, and to undertake a full entomological study of the possibility of transmission by household arthropods.

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